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14. ABSTRACT

Using a homology-based bioinformatics approach, a new structural model of the vaccinia virus (VV) I7L proteinase active site has been generated. This model was used to perform Virtual Ligand Screening of a comprehensive library of approximately 3.5 million available compounds including about 208,000 available ketones and aldehydes. Compounds with a docking score of <-32 were ordered and screened using our newly developed fluorescence quench biochemical assay for those compounds able to inhibit the activity of the I7L enzyme. Compounds have been identified that inhibit I7L more than 50% at 200 µM which validate the 3D ligand binding model and provide initial leads for further rational optimization of poxvirus I7L proteinase inhibitors.

15. SUBJECT TERMS
Smallpox, cysteine proteinase, antiviral drug, HTS-screening, rational drug design, vaccinia virus, I7L

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Introduction

Smallpox virus is considered one of the most significant threats for use as a biowarfare agent. Due to complications from vaccination, mass immunization of the populace is contra-indicated. Our current research seeks to develop effective anti-poxvirus drug(s). Using vaccinia virus (VV) as a model system, the goal of our currently funded work is to determine whether the I7L cysteine proteinase or the G1L metalloproteinase encoded by VV is the poxvirus core protein proteinase (vCPP) that is essential for viral maturation and production of infectious progeny. We have recently demonstrated that the I7L cysteine proteinase is the vCPP (Byrd et al., 2002) and have continued to study the role of this enzyme during virus growth (Byrd et al., 2003; 2004; 2005a; 2005b). Given this information however, we are also positioned to launch a concerted effort to identify and develop I7L inhibitors as candidate antiviral drugs. The specific goals of the experiments outlined in this report are to: 1) Over-express and purify enzymatically-active I7L proteins; 2) Develop both biochemical and tissue culture assays to measure I7L activity; 3) Utilize a combination of rational drug design and high throughput screening procedures to identify potential I7L inhibitors; and 4) To test candidate inhibitors for their ability to inhibit poxvirus replication in infected cells and appropriate animal models. Successful completion of these experiments will identify I7L inhibitors that can be advanced into pre-clinical and clinical development as antiviral drugs. Such drugs will be an essential addition to our pharmaceutical armamentarium against the deliberate or accidental introduction of a pathogenic poxvirus into our environment in order to protect members of the armed forces or the general populace.

Body

1. **Expression and purification of vCPP (I7L).** SIGA has utilized many different systems to try to express enzymatically active I7L protein including numerous *E. coli* based expression systems, SIGA's proprietary SPEX and PLEX systems, mammalian cell expression, Baculovirus, and yeast. Some success has been seen with the Baculovirus and yeast systems so efforts were extended to bacterial expression with various recombinant constructs. I7L was cloned into the pBAD bacterial expression vector with its native N-terminus and a C-terminal 6x-his tag. Both I7L and mutant-I7L have been expressed from this system as soluble protein in the supernatant as well as some residual material in the insoluble pellet. Initial studies to test for activity of the enzyme using our fluorescence quench assay have shown activity of I7L. In addition to bacterially expressed I7L, enzyme expressed through mammalian cell extracts can be used in each of the cleavage assays to verify activity and inhibition of activity.

2. **Cleavage assays.** SIGA has developed three separate cleavage assays as well as a cell based whole virus assay to test for the ability of specific compounds to inhibit the cleavage activity of I7L and thus inhibit the virus. The first cleavage assay is a *trans*-processing assay in which I7L and its substrate are expressed in mammalian cells from a plasmid based system,

compounds are added to the cell medium, and cleavage of the substrate is measured through Western-blot analysis (Figure 1).

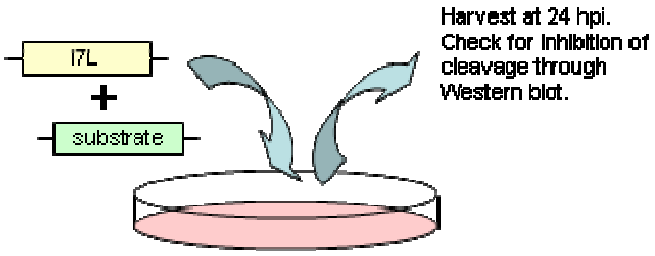


Figure 1. *Trans*-processing cleavage assay.

The second cleavage assay is an *in-vitro* TNT cleavage assay. I7L from infected cell extracts is used as the source of enzyme and P25K, P4a, or P4b produced in a transcription and translation kit is used as substrate. These are mixed in the presence or absence of inhibitor and cleavage is monitored through Western-blot analysis. Compounds that inhibit cleavage will show only the precursor P25K band and no cleavage product (Figure 2).

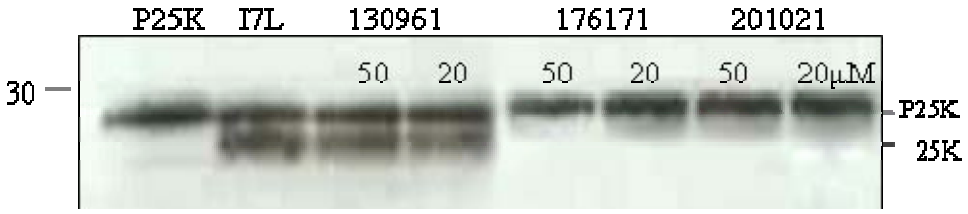


Figure 2. *In-vitro* TNT cleavage assay

The third cleavage assay is a fluorescence quench biochemical assay based on a fluorescence-quench pair of probes that are conjugated to the amino and carboxy-terminal ends of an I7L cleavage peptide respectively. When the quench group is in close proximity to the fluorescent group, prior to cleavage, there is a relatively low or baseline signal. Following cleavage of the peptide by the I7L protease, the quench group is no longer in close proximity to the fluorescent group and an increase in fluorescence is measured through a plate reader. Compounds that inhibit the activity of I7L result in a low fluorescence reading (Figure 3). This assay has the benefits of being suitable for a 96-well plate format, using relatively small concentrations of inhibitors, and requiring hours instead of overnight to read so can be used for high-throughput screening capabilities.

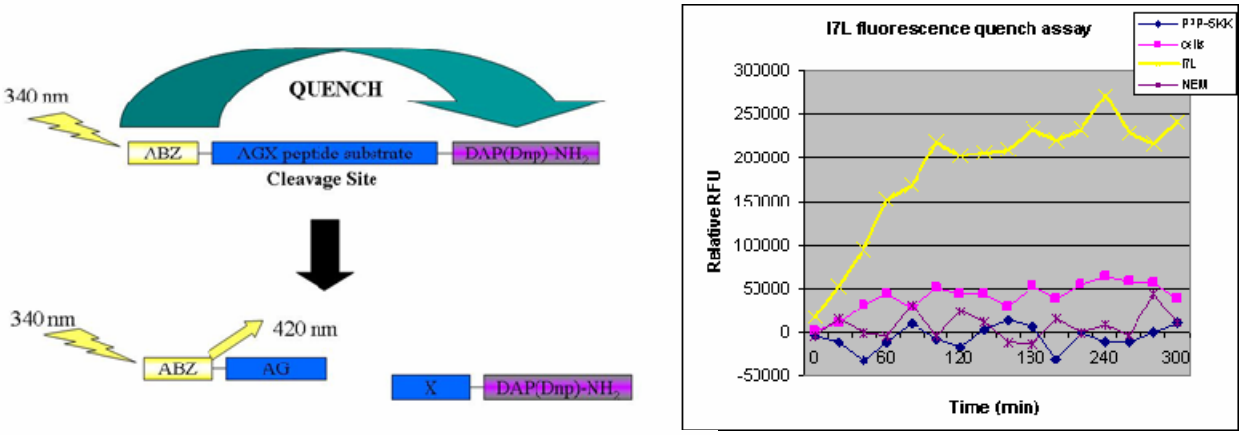
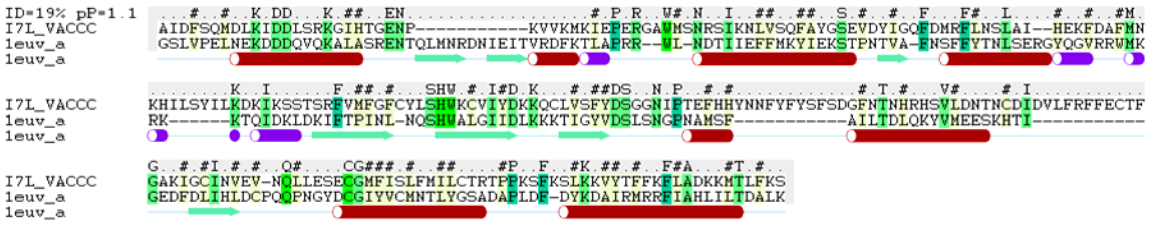


Figure 3. Diagram of the fluorescence quench assay along with a measurement of I7L specific activity measure by an increase in fluorescence.

The fourth assay is a cell based assay where mammalian cells in tissue culture plates are infected with vaccinia virus expressing a green-fluorescent protein, compound is added, and the ability of the virus to replicate is measured through a reduction in fluorescence as well as a reduction in plaque formation. This assay has the benefits of being able to determine whether a compound will be able to get inside the cell to inhibit the virus, as well as provide toxicity information on the cells themselves.

3. **Rational drug design.** A 3D model of the I7L protein was built based on the yeast Ulp1 cysteine proteinase structural template (PDB: 1euv), the closest homologue of I7L in the PDB database (Figure 4). The predicted active site of I7L represents a tight binding pocket with a volume of about 400 $Å^3$, which is suitable for rational drug design. While the core of this binding pocket is fully conserved between I7L and the mitochondrial protease Ulp1 in humans, the difference in non-conserved residues Glu325 and Gly260 should be sufficient to allow design of I7L selective inhibitors.

A)



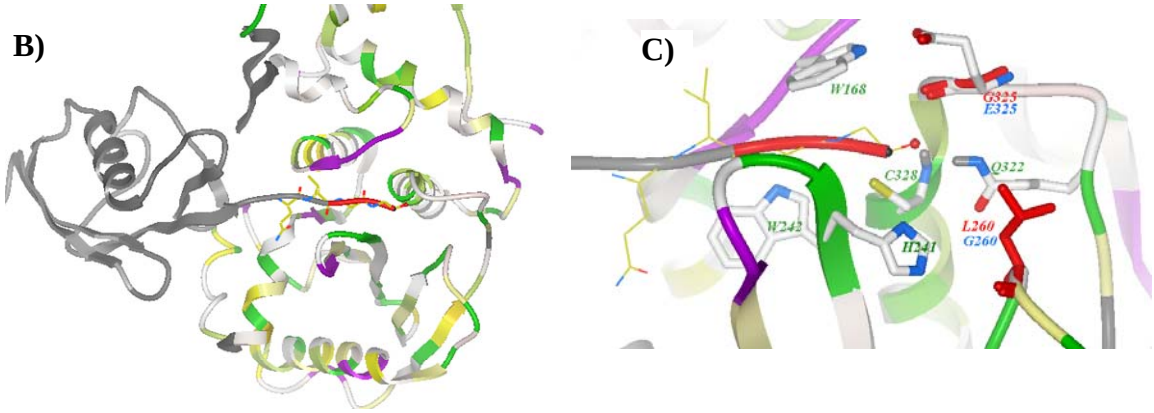


Figure 4. Structural model of I7L and its active site, base on X-ray structure of Ulp1 cysteine protease (PDB code 1euv) **A)** Sequence-structure alignment between I7L and Ulp1. **B)** Predicted structure of the I7L proteinase, with ribbon representing residue identity with Ulp1 (green is fully conserved, yellow are similar residues). Structure of Ulp1 substrate is shown as grey ribbon with two C-terminal residues highlighted in red. **C)** Close up of I7L model active site. Five conserved residues are shown as sticks colored by atom type with green labels. Two residues of the I7L active site (G325 and L260) that differ from Ulp1 are shown in red sticks.

4. **In vitro and in vivo screening for inhibitor activity.** Candidate I7L inhibitors were predicted using the Molsoft ICM-virtual ligand screen. The standard ICM VLS procedure was modified to reflect covalent binding of inhibitors to the active site Cys328 of I7L. Compounds were selected from a non-redundant library of ~3.5M compounds available from more than 40 vendors, as well as from our in-house diversity library of ~208K compounds. These compounds were divided into 4 groups based on their chemical reactivity with the I7L active site thiol: Aliphatic Aldehydes (AL(al), Aromatic Aldehydes (AL(ar), Aliphatic Ketones (KE(al) and Aromatic Ketones (KE(ar)). Aldehydes are considered to be the most reactive, followed by aliphatic ketones, and least reactive are the aromatic ketones. Compounds with a docking score of <-32 were ordered and screened through the fluorescence quench assay for their ability to inhibit I7L enzyme.

Key Research Accomplishments

- Expressed active I7L from mammalian cell extracts for use in biochemical assays.
- Expressed and purified I7L from bacteria for use in biochemical assays and high throughput screening.
- New rational model of I7L active site constructed
- Virtual ligand screening done on ~3.5 million available compounds for those predicted to fit into the I7L active site pocket.
- 456 rationally designed compounds with a docking score of <-32 were tested in house for inhibitory activity

Reportable Outcomes (manuscripts, abstracts, presentation, patents, etc)

Manuscripts

1. Hruby, D.E., and C.M. Byrd. 2006. Less is more: essential role of poxvirus proteinases during assembly. Microbe 1:70-75

2. Honeychurch, K.M., C.M. Byrd, and D.E. Hruby. 2006. Mutational analysis of the potential catalytic residues of the VV G1L metalloproteinase. Virology Journal 3:7

3. Byrd, C.M., and D.E. Hruby. 2006. Vaccinia virus proteolysis – a review. Reviews in Medical Virology 16:3

4. Byrd, C.M. and D.E. Hruby. 2006. Viral proteinases – targets of opportunity. Drug Development Research 67:501-510

5. Moerdyk, M., C. M. Byrd, and D. E. Hruby. 2006. Analysis of vaccinia virus temperature sensitive I7L mutants reveals two potential functional domains. Virology Journal 3:64

Abstracts & Presentations

1. Bolken, T., G. Yang, Y. Chen, C. Harver, C. M. Byrd, K. Jones, D.E. Hruby, R. Andrews, R. Jordan. 2006. TTP-855: A potent and specific inhibitor of poxvirus replication. XVIth International Poxvirus & Iridovirus Workshop, Indian Wells, California

2. Moerdyk, M.J., C.M. Byrd, and D.E. Hruby. 2006. Characterization of vaccinia virus I7L temperature sensitive mutants. American Society for Virology, Madison, WI

3. Honeychurch, K.M., C.M. Byrd, and D.E. Hruby. 2006. Mutational analysis of the potential catalytic residues of the vv G1L metalloproteinase. American Society for Virology, Madison, WI

4. Honeychurch, K.M., C.M. Byrd and D.E. Hruby. 2006. Mutational analysis of the potential catalytic residues of the VV G1L metalloproteinase. OSU Graduate Student Poster Symposuim. Corvallis, OR. January 26.

5. Honeychurch, K.M. and D.E. Hruby. 2006. Subcellular localization of vaccinia virus G1L within infected cells. FASEB Summer Research Conference on Poxviruses. Indian Wells, CA. June 3-8, 2006

6. Stavale, E.J., C.M Byrd, R. Jordan, and D.E. Hruby. 2007. Expression and purification of vaccinia virus I7L from E. coli. American Society for Virology, Corvallis, OR

Patents

Screening Method for Orthopoxvirus Antivirals (US & PCT applications)

Conclusions

Using a homology-based bioinformatics approach, a new structural model of the vaccinia virus (VV) I7L proteinase active site has been generated. This model was used to perform Virtual Ligand Screening of a comprehensive library of approximately 3.5 million available compounds including about 208,000 available ketones and aldehydes. Compounds with a docking score of <-32 were ordered and screened using our newly developed fluorescence quench biochemical assay as well as in a cell based assay against the whole virus, for those compounds able to inhibit the activity of the I7L enzyme.

A cell based whole-virus assay, an *in-vitro trans*-processing assay, an *in-vitro* TNT cleavage assay, and a fluorescence-quench biochemical assay were developed to be able to determine the activity as well as the toxicity of potential inhibitors. These assays can be utilized individually or in combination to be able to screen compounds either in a high-throughput format, or low-throughput for individual rationally-deisgned compounds.

SIGA has identified compounds that inhibit I7L by more than 50% at 200 µM which validate the 3D ligand binding model and provide initial leads for further rational optimization of poxvirus I7L proteinase inhibitors.

References

1. Byrd, C. M., T. C. Bolken, and D. E. Hruby. 2002. The vaccinia virus I7L gene product is the core protein proteinase. J Virol. 76:8973-6

2. Byrd, C.M., T.C. Bolken, and D.E. Hruby, 2003. Molecular dissection of the vaccinia virus I7L core protein proteinase. J. Virol 77:11279-11283.

3. Byrd, C. M., T. C. Bolken, A. M. Mjalli, M. N. Arimilli, R. C. Andrews, R. Rothlein, T. Andrea, M. Rao, K. L. Owens and D. E. Hruby. 2004. New Class of Orthopoxvirus Antiviral Drugs That Block Viral Maturation. J. Virol 78:12147-12156.

4. Byrd, C.M., and D.E. Hruby. 2005. A conditional-lethal vaccinia virus mutant demonstrates that the I7L gene product is required for virion morphogenesis. Virology Journal 2:4.

5. Byrd, C.M., and D.E. Hruby. 2005. Development of an *in vitro* cleavage assay system to examine Vaccinia virus I7L cysteine proteinase activity. Virology Journal 2:63.

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Less Is More: Poxvirus Proteolysis

Two proteinases cleave each of the three major poxvirus core proteins, helping to regulate the assembly of infectious viral particles

Dennis E. Hruby and Chelsea M. Byrd

Before progeny virus particles depart an infected cell, each virion needs to contain viral nucleic acid, accessory proteins, and enzymes within an outer shell before the capsid structure is completed to shield the sensitive cargo from the external environment. One mechanism that many viruses use to govern this process is morphogenic proteolysis.

During the late stages of replication, immature virions are assembled, into which all the essential viral components are packaged. Then at the proper time and place, virus-encoded proteinases cleave viral structural proteins, launching a series of structural rearrangements that yield the mature infectious viral particle, whose genome is protected and which is ready to be transported to the next susceptible host. Morphogenic proteolysis is crucial for simple RNA viruses such as poliovirus and HIV, and also appears to play a central role in the assembly of more complex DNA viruses such as the poxviruses.

- During the final stages of replication for many viruses, proteinases cleave structural proteins that rearrange before yielding mature viral particles.
- Three major vaccinia virus core proteins, which are products of the A10L, A3L, and L4R genes and make up about one-third of the dry weight of the virion, each derive from higher molecular weight precursors.
- The vaccinia virus G1L gene product appears to be the first example of a virus-encoded metalloproteinase, while the I7L gene product exhibits sequence homology to several cysteine proteinases; both enzymes appear to be needed for viral core protein processing.

Poxvirus Replication Is Complex

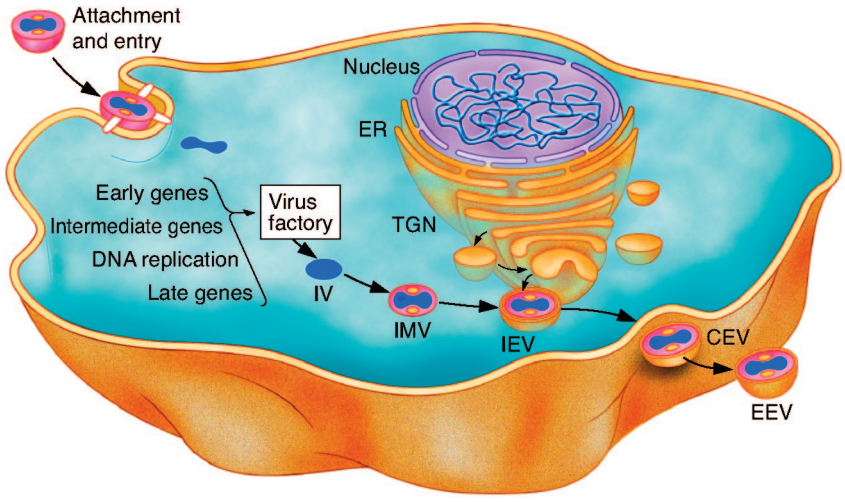
The assembly of poxviruses, such as vaccinia virus, is a complex, extremely well-regulated process. Approximately 200 viral gene products are expressed in a temporally regulated fashion during the replication cycle, culminating in infectious progeny virions within 10–12 hours. First, enveloped virions bind to susceptible cells, then penetrate the cytoplasm, losing their outer membrane and liberating the viral core, which contains the viral genome, structural proteins, and the viral transcriptional apparatus, including RNA polymerase, capping enzyme, and polyA polymerase (Fig. 1).

Viral early genes, approximately half the viral genome, are transcribed from the intact viral core. Once those early genes are expressed, the viral core breaks down, liberating the viral genome and expressing a second, or intermediate, set of viral gene products. Their appearance advances the virus into the late stage of its life cycle, during which viral DNA is replicated and late-gene products are expressed in perinuclear cytoplasmic virus-producing complexes. All the essential starting materials for progeny virus particles are assembled into a complex that buds through the intermediate compartment to produce spherical noninfectious particles (IV, immature virus).

Each of these particles then undergoes a series of condensations and rearrangements to produce infectious particles (IMV, intracellular mature virus) having the hallmark biconcave core and lateral bodies that are observed by thin-section transmission elec-

Dennis E. Hruby is a professor in the Department of Microbiology at Oregon State University, Corvallis, and Chelsea M. Byrd is a former graduate research associate who is now a scientist at SIGA Technologies, Inc., in Corvallis.

FIGURE 1



Poxvirus life cycle.

tron microscopy. Some of the IV particles acquire two additional membranes from the host *trans*-Golgi to become triple-wrapped intracellular enveloped virus (IEV), with the outermost envelope being lost by membrane fusion as the particle exits the cell to become cell-associated virus (CEV) or extracellular enveloped virus (EEV). Although the details of this morphogenesis pathway are being investigated, we know that morphogenic proteolysis plays a major role in the pathway, helping to convert IV to fully infective IMV particles.

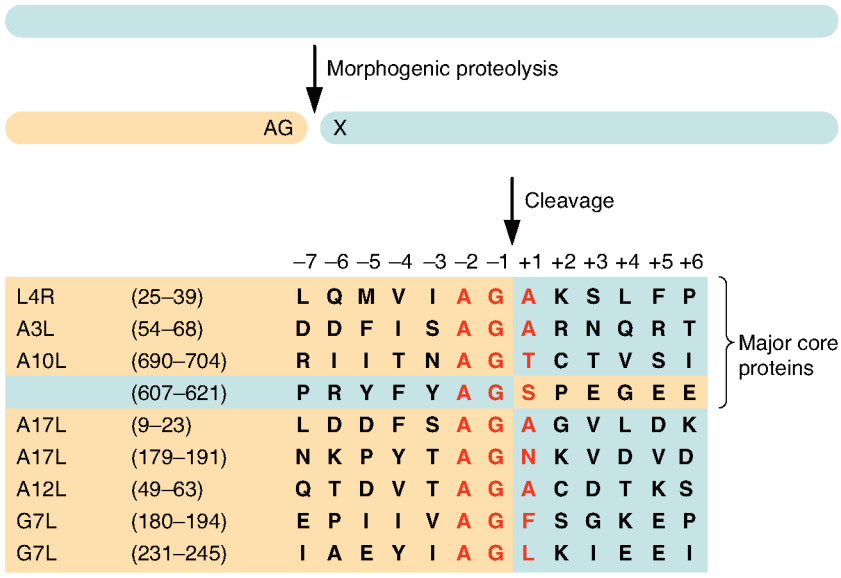
Cleaving the Building Blocks

Because the sizes of the late-phase major poxvirus proteins do not match those of the major virion structural proteins, investigators long suspected that proteolytic processing occurs during virus assembly. Pulse-chase labeling and peptide mapping procedures subsequently confirmed that the three major VV core proteins (A10L, A3L, and L4R gene products) that make up about one-third of the dry weight of the virion all derive from higher-molecular-weight precursors.

The abundance of these proteins in viral particles enables investigators to purify and subject them to N-terminal microsequencing. This analysis reveals that both A3L and L4R are processed by a single endoproteolytic cleavage within an amino-terminal proximal site containing the tripeptide AGA. The 66-kDa A3L precursor is cut between residues 61 and 62 to produce a 60-kDa product, while the 28-kDa L4R precursor is cut between residues 32 and 33 to produce a 25-kDa product. In contrast, the A10L precursor, which is more slowly processed in vivo, is cleaved at two sites within the protein, between residues 613 and 615 and between residues 696 and 698.

Although the mature A10L, A3L, and L4R proteins are major virion constituents, none of

FIGURE 2



Processing of vaccinia virus core proteins.

Early Detour from Marine Biology Led Hruby to Microbiology

Dennis Hruby arrived at Oregon State University (OSU) in 1969 intending to study marine biology, not realizing it was a graduate program. Undeterred, he opted to study microbiology.

During his junior year, while taking a course from John L. Fryer, longtime professor and chair of the microbiology department, Hruby protested the 98% score he received on his midterm exam. "I thought I deserved 100, so I turned back my exam with a written explanation why," he recalls. "A couple of days later, as I was walking to class, I heard Dr. Fryer call my name. I was sure he was going to read me the riot act. Instead, he recruited me into his research lab where I spent the next two years, and fell in love with microbiology research."

Today Hruby is a professor of microbiology at OSU, as well as the chief scientific officer of SIGA Technologies, Inc., a biotechnology company seeking novel treatments for infectious diseases, particularly those that might be used for biowarfare or terrorism. He has studied poxvirus replication in his academic lab for the past 28 years, also with the aim of developing potential drugs. He splits his time between the two jobs, which he finds "synergistic."

"My private sector activities are focused on driving product candidates toward the clinic and continually challenge me to learn new things that were never taught in my graduate school curriculum," he says. "The job is high stress and fast paced. In contrast, my academic activities are a little more routine, but equally rewarding. The opportunity to pursue whatever interests me and interact with and teach bright young students is a real privilege."

Hruby, 54, was born in Loup City, Neb., and lived on a farm there until he was 10, when his family moved to Oregon. Neither of his parents went to college, but they "set an example with a tremendous work ethic and a can-do attitude," he says. "I was allowed to pursue whatever I wanted to—provided all my chores were done first." Hruby cannot say what led him to become a scientist—only what didn't. "I can guarantee that working the graveyard shift at the plywood mill during my college summers taught me what I didn't want to do for a career," he says.

He received a B.S. in microbiology in 1973 and, abandoning his earlier plan to study marine biology, continued with doctoral studies in microbiology at the University of Colorado Medical Center, where he received his Ph.D. in 1977. He returned to OSU in 1983 after postdoctoral stints at the State University of New York at Stony Brook and the University of Wisconsin. He also served as a visiting scientist at the Massachusetts Institute of Technology and as assistant professor at the University of Texas, Austin.

Hruby lives with his wife and two children, ages 17 and 15, on a horse ranch. "There's nothing like cleaning a few stalls in the morning to prepare you for dealing with the rest of the day," he says. He rises at 4:30 a.m. to work out for an hour "to stay in shape for golf, racquetball, and skiing." But his favorite leisure activity is fishing. "I have a boat and get out on the lakes and ocean as often as possible," he says. "My choice of seminar guests to invite is usually dictated by whether they like to go fishing."

He also takes pleasure from his interactions with graduate students and colleagues. "We now carry out most of our best discussions on the golf course," he says. "Life, like most of our drives, is short."

Marlene Cimons
Marlene Cimons is a freelance writer in Bethesda, Md.

the liberated small peptides has ever been identified. Not finding them could indicate that they are either degraded or extruded from the virion. However, their small size and acidic nature suggest that they might become complexed with the viral DNA to facilitate genome condensation.

In any case, the major VV core proteins are processed at AG(X) sites. Detailed mutagenesis studies confirm the importance of amino acid residues in the AG(X) site as well as protein characteristics within the excised peptide. Furthermore, these studies demonstrate that the core proteins are expressed as a precursor to be packaged into the virion. If mature proteins lacking the cleaved peptide are expressed from plasmids, they remain cytoplasmic and do not become virion-associated, suggesting correct targeting requires structural or sequence attributes of the precursor proteins.

The VV genome sequence contains 82 AGX

motifs within the predicted VV proteome, but processing of only some of those motifs has been experimentally verified (Fig. 2). Some VV proteins contain AGA motifs that are not processed, including those found in the VV DNA polymerase, which is an early gene product, and in F13L, a major constituent of the outer EEV membrane. From these studies, we surmise that processing is "contextual," meaning that VV proteins containing the AGX motif are processed only if they are late proteins and virion core constituents. If so, the precursor apparently is packaged into the IV particle and cleaved when IV is converted to the IMV particle.

Drugs such as rifampicin or temperature-sensitive mutations affecting morphogenesis do not interfere with packaging of the precursor proteins but abrogate morphogenic processing.

FIGURE 3

I7L	G1L
47 KDa protein	67 KDa protein
Essential late gene product	Essential late gene product
Conserved in all poxviruses	Conserved in all poxviruses
Homologous to C57 family of cysteine proteinases	Homologous to M44 family of metalloproteinases
Predicted catalytic triad (H, D, C)	Predicted HXXEH active site
VV core protein proteinase	Biological function unknown

Poxvirus proteinases.

A Tale of Two Proteinases

Even after recognizing that these poxvirus core proteins are subject to morphogenic proteolysis, the responsible proteinases remained elusive until recently. Many other viruses encode and express proteinases, making it more likely that vaccinia virus with its large genome would follow suit. Moreover, this virus has a broad host range, but no host proteinases are known to recognize and cleave the AGX motif. However, our efforts to identify vaccinia proteinases by testing extracts of virions or virus-infected cells for either core protein precursors or peptides containing AGX cleavage sites proved unsuccessful.

Bioinformatics provided a more fruitful pathway for identifying two proteinases that could be responsible for processing the poxvirus core proteins (Fig. 3). One of them, the gene product of the VV G1L open reading frame, appears to encode a metalloproteinase, based on the presence of an inverted HXXEH metal-binding site and conserved downstream ENE motif, similar to one found in the thermolysin subfamily of metalloproteinases. We also know that the 67-kDa G1L protein is expressed late during viral infection and that the gene is highly conserved among the poxviruses. Further, despite little primary sequence homology with other proteins, structural threading models reveal a striking structural resemblance to the yeast mitochondrial processing peptidase (MPP), a known metalloproteinase.

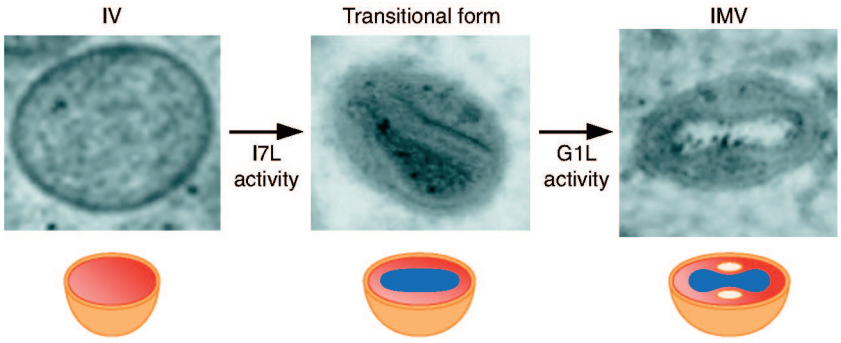
G1L appears to be the first example of a virus-encoded metalloproteinase. All other known viral proteinases belong to the cysteine, serine, or aspartic acid classes of proteinases.

We also identified a second candidate poxvirus proteinase, the product of the VV I7L "late" gene, which is expressed as a 47-kDa protein. Like G1L, the I7L gene sequence is highly conserved among poxviruses. The I7L gene product exhibits sequence homology to several cysteine proteinases, including those expressed by members of two other families of DNA viruses, the adenoviruses and asfarviruses, which includes the African swine fever virus (ASFV). Sequence similarities were highest in the region flanking the putative active site of the I7L enzyme, containing histidine, cysteine, and aspartic acid.

Both Proteinases Appear Necessary for Viral Core Processing

Our bioinformatics-based approach uncovered two poxvirus proteinases, but which is the key player? The answer appears to be both.

FIGURE 4



Morphogenic proteolytic cascade during poxvirus maturation.

Because a biochemical approach to studying VV proteolysis continues to be elusive, we instead analyzed infected cells with a temperature-sensitive I7L mutant at the nonpermissive temperature, 40°C. Although all the viral proteins are made, including the viral core protein precursors, processing does not occur and no infectious progeny form. However, when plasmids capable of expressing either I7L or G1L are introduced, I7L proves to be a key enzyme for restoring processing. Subsequently we identified the likely active site residues in I7L, verified that cleavage occurs at the correct site within the AGX motif, and demonstrated that I7L cleaves each of the three major core protein precursors, A3L, A10L, and L4R. Thus, like its adenovirus and ASFV homologs, the I7L gene product appears to be the viral core protein proteinase.

What about G1L? Additional insights into the roles and relationships of the I7L and G1L proteinases emerged through our analysis of conditional-lethal I7L and G1L VV mutants. For instance, if I7L activity is repressed, the VV infection proceeds normally through formation of IV, but without core protein processing and condensation. However, if G1L activity is repressed, IV particles form, core protein precursors cleave, and virion core condensation initiates but is not completed.

These results suggest that both I7L and G1L are needed to complete morphogenic proteolysis (Fig. 4). We hypothesize that during poxvirus assembly, immature viral particles contain all the VV components necessary to produce an infectious virion, including viral DNA, enzymes, and structural proteins with the core proteins in their precursor form. Virion loading and primary envelopment are somehow signaled to the I7L cysteine proteinase, which then cleaves the major structural proteins and initiates core condensation. At this stage, G1L is activated to complete morphogenic processing and produce mature infectious virions. This proteolytic processing cascade could help to ensure that viral assembly is complete and ordered.

Several key questions remain to be answered regarding poxvirus proteolysis. What is the nature of the active I7L and G1L enzymes? Do they act as monomers or multimers? Do they require cofactors or posttranslational modifications for activity? What are the virion substrates of G1L? Does it act on a distinct subset of the AGX motifs, or does G1L cleave a completely different set of proteins? What signals activate the I7L and G1L enzymes at the correct time and place? Answering these questions will depend on establishing in vitro enzyme assays for both enzymes.

Finally, both the I7L and G1L gene products are highly conserved and appear to be essential for virus replication, suggesting that they are potentially important targets for designing antiviral drugs with which to prevent or treat diseases caused by orthopoxviruses such as smallpox or sylvatic monkeypox.

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Short report

Mutational analysis of the potential catalytic residues of the VV G1L metalloproteinase

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Abstract

The vaccinia virus G1L open-reading frame is predicted to be a metalloproteinase based upon the presence of a conserved zinc-binding motif. Western blot analysis demonstrates G1L undergoes proteolytic processing during the course of infection, although the significance of this event is unknown. In order to determine which amino acid residues are important for G1L activity, a plasmid-borne library of G1L constructs containing mutations in and about the active site was created. Transient expression analysis coupled with a *trans* complementation assay of a conditionally-lethal mutant virus suggest that, of the mutants, only glutamic acid 120 is non-essential for G1L processing to occur.

Findings

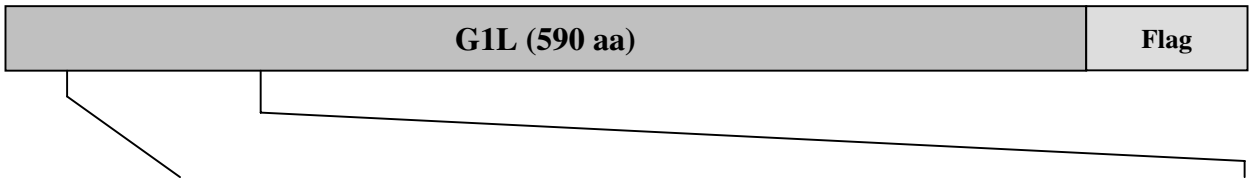
Vaccinia virus (VV) is among the largest of the DNA viruses and represents the prototypic member of the Orthopoxvirus genus. It is an enveloped virus and possesses a linear double-stranded genome containing greater than 200 mostly non-overlapping open reading frames. Throughout its life cycle, VV replicates exclusively in the cytoplasm of infected cells, although the presence of a nucleus is required in order for the virus to mature properly [1,2]. During replication, VV undergoes three distinct stages of gene expression, the products of which are referred to as early, intermediate and late proteins. In general, early proteins are components of the replication machinery, intermediate proteins assist in the transcription of late proteins and late proteins consist of the virion structural elements, the trafficking and assembly of which are regulated by modifications such as acylation, myristoylation and palmitoylation [3-8] as well as by host cell and virally encoded proteases.

The complete DNA sequence of VV revealed the presence of two potential proteinases, the products of the I7L and G1L open reading frames [9]. The first, I7L, was originally identified by limited sequence similarity to a ubiquitin-like proteinase in yeast [10]. I7L is now recognized as the VV core protein proteinase and is at least one of the entities responsible for initiating the morphogenic transformation of immature virus (IV) particles into intracellular mature virus (IMV) [11-13] and is currently a target of rational antiviral drug design [14]. The second apparent proteinase is G1L. G1L was initially thought to be the proteinase responsible for the late-stage proteolytic morphogenesis of at least one of the viral core proteins based upon results obtained from a transcriptionally controlled *trans*-processing assay [15] G1L contains a canonical HXXEH zinc-binding motif [16], which is a direct inversion of the established HEXXH motif found in a wide array of matrix metalloendopeptidases (MMPs), including thermolysin [17], aminopeptidase N [18] and collagenase [19]. The particular sequence contained within

A

Putative metalloprotease G1L [Vaccinia virus]	34/D**E**ILGIA**H**LL**EH**LLISF
Chain B, Yeast Mitochondrial Processing Peptidase	44/VKNNGTA**H**FL**EH**LAFKG
Insulinase-like peptidase M16 [*Pelobacter propionicus*]	79/D**E**R-GIA**H**LL**EH**MLFKG
Peptidase, M16 family [*Campylobacter jejuni*]	38/N**E**IMGIA**H**ML**EH**LNFKS
Hypothetical zinc protease [*Leptospira interrogans*]	97/P**E**IAGTA**H**LL**EH**MLFKG
Protease (pqqE) [*Campylobacter coli*]	38/N**E**IMGIA**H**ML**EH**LNFKS
Putative zinc protease [*Helicobacter hepaticus*]	54/N**E**VMGIA**H**ML**EH**L--SF
Predicted Zn-dependent peptidases [*Microbulbifer degradans*]	76/YGETGMA**H**LL**EH**LLFKG
Potential a-pheromone maturation protease [*Candida albicans*]	70/MNYLGLT**H**LL**EH**LIIFN

B



VV(WR)	30/ENDID**E**ILGIA**H**LL**EH**LLISF/50	107/HIK**E**L**E**N**E**YYFRN**E**VFH/123
E35A	30/ENDID**A**ILGIAHLLEHLLISF/50	107/HIKELENEYYFRNEVFH/123
H41A	30/ENDIDEILGIA**A**LLEHLLISF/50	107/HIKELENEYYFRNEVFH/123
E44A	30/ENDIDEILGIAHLL**A**HLLISF/50	107/HIKELENEYYFRNEVFH/123
H45A	30/ENDIDEILGIAHLLE**A**LLISF/50	107/HIKELENEYYFRNEVFH/123
E110A	30/ENDIDEILGIAHLLEHLLISF/50	107/HIK**A**LENEYYFRNEVFH/123
E112A	30/ENDIDEILGIAHLLEHLLISF/50	107/HIKEL**A**NEYYFRNEVFH/123
E114A	30/ENDIDEILGIAHLLEHLLISF/50	107/HIKELEN**A**YYFRNEVFH/123
E120A	30/ENDIDEILGIAHLLEHLLISF/50	107/HIKELENEYYFRN**A**VFH/123

Figure 1
(A) Alignment of the VV G1L putative catalytic domain with the catalytic domain found throughout MMPs. (B) G1L mutant library. Schematic of alanine substitutions within the G1L ORF. Each construct includes a C-terminal Flag epitope for detection by Western blot analysis.

G1L is characteristic of the M16 (pitrilysin) family of MMPs as well as a variety of proteins found in bacteria and yeast (Fig. 1A). Most known MMPs include a signal sequence to allow for secretion, an inhibitory pro-sequence to regulate activity and a catalytic domain containing a catalytically active Zn^{2+} ion [20]. Amino acid sequence analysis demonstrated very little similarity between G1L and other MMPs, aside from the presence of a potential zinc-binding motif. However, computational modeling has revealed that structurally, G1L appears to contain a significant likeness to the β-subunit of the yeast mitochondrial processing peptidase (MPP), which is the

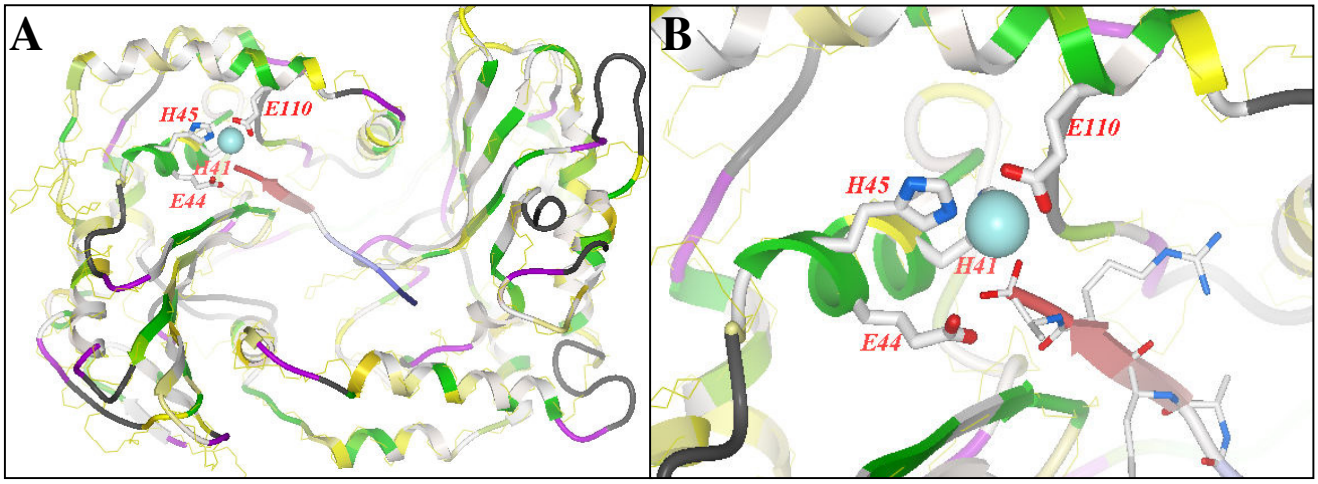


Figure 2
Homology model of G1L using the yeast MPP as a template. G1L is depicted as a ribbon colored by alignment: identical residues, green; similar residues, yellow; non-conserved residues, white; insertions, magenta; deletions, black. The MPP template is shown as a thin yellow line. Active site residues are represented as thick sticks and the Zn^{2+} ion as a cyan sphere. A substrate peptide is shown as blue and red ribbon and thin sticks. (A) G1L in its entirety. (B) Close-up view of the putative active site residues.

closest structural homolog with an available X-ray structure [PDB:1hr9.b] (Fig. 2) [21,22]. The β-subunit of the yeast enzyme is characterized by the presence of an inverted zinc-binding motif, $HXXEHX_nE$, where two histidine residues and a distal glutamic acid (E) residue coordinate a zinc cation [16,23,24]. The E residue within the HXXEH motif is involved in peptide bond hydrolysis through the activation of water [23,25]. The essential downstream E residue for M16 MMPs is found within a region containing several completely conserved E residues [23]. Although G1L lacks an exact match to this region, it does contain a region 65 residues downstream of the HXXEH sequence consisting of $ELENEX_5E$ (residues 110 through 120) that is very highly conserved among poxviruses.

While it is tempting to predict that VV G1L behaves in a manner similar to yeast MPP, the fact remains that very little is actually known about G1L activity. Through the development of a conditional-lethal recombinant vaccinia virus, G1L was identified as an essential component of the VV replication cycle [26,27]. Conditional-mutants grown under non-permissive conditions arrested their replication subsequent to core protein cleavage but prior to complete core condensation suggesting the major viral proteins are expressed and processed independently of G1L but that G1L plays a crucial role in the conversion of vaccinia virus from immature virions into infectious IMV particles [26-28]. Western blot analysis suggests G1L exists initially as a 68 kDa entity, which may be cleaved into 46 kDa and 22 kDa products [26]; however, the significance of this cleavage remains unclear. The presence of a bound zinc ion has yet to be experimentally confirmed, although efforts to obtain G1L in sufficient quantities and of sufficient purity to allow for such analyses are currently underway.

In the present study, an analysis of the putative active site of G1L was carried out in an attempt to understand which amino acid residues are important for G1L processing as well as which of the four downstream E residues present within the highly conserved $ELENEX_5E$ sequence is likely to participate in the coordination of a zinc ion if such an interaction does in fact occur. Through the use of a library of transiently expressed G1L mutants containing C-terminal flag epitopes coupled with rescue analysis of a tetracycline-dependent conditional-lethal mutant, the results obtained suggest that only E120 can withstand mutation and still produce a phenotype similar to what is observed for wild type G1L.

The putative active site of VV G1L is thought to consist of histidine (H) residues 41 and 45 and a downstream E, all of which are thought to contribute to zinc-binding, and E residue 44, which is predicted to participate in the hydrolysis of the substrate peptide bond. In this study, each of these residues was systematically mutated to alanine (A) (Fig 1B). There are four candidate downstream E residues, including E110, E112, E114 and E120. Computational modeling utilizing the β subunit of the yeast MPP suggests

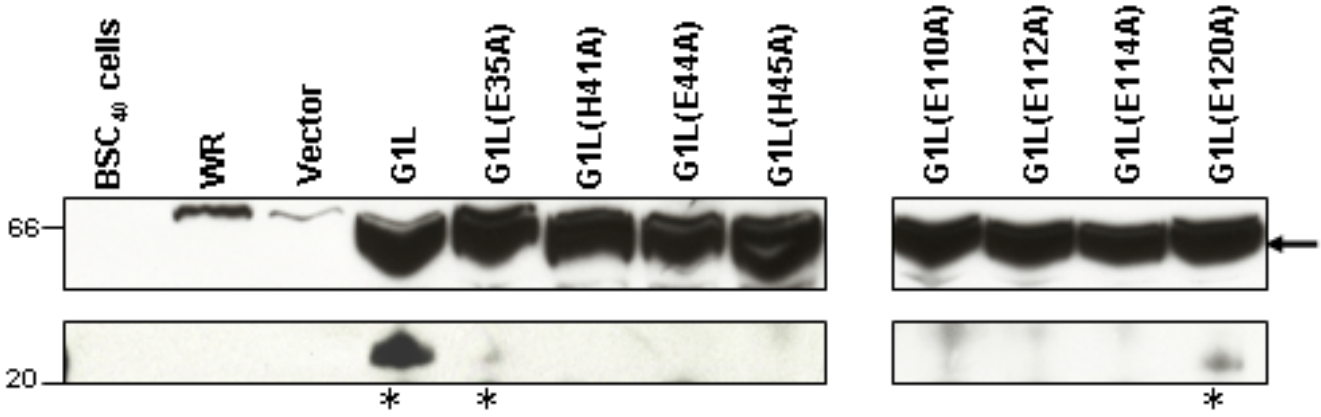


Figure 3
G1L undergoes proteolytic processing. BSC_{40} cells were transfected with 1.5 μg of plasmid DNA containing either wild type G1L or one of the eight single-site mutants. Four hours later, the transfection solution was removed and cells were infected with VV strain WR at an MOI of two. Twenty-four hours later, cells were harvested and extracts were subjected to Western blot analysis with anti-Flag antisera. The top panel demonstrates full length G1L, indicated by the arrow, and the lower panel shows the resulting cleavage products indicated by *.

E110 is the downstream E residue involved in zinc coordination (Fig. 2). Mutation of E112 or E114 was shown to abrogate the processing of p25K in a *trans*-processing assay [15], although whether or not this result was an artifact of the assay remains to be determined. The fact that both E112 and E114 are very highly conserved throughout the Orthopoxvirus genus as well as poxviruses in general implies that at least one of them may be involved in the activity of G1L. Glutamic acid residue 120 was selected for mutation since its location 74 residues downstream from the HXXEH motif follows the same pattern observed for members of the zinc-dependent M16 family of metalloproteinases [29,30] as well as a variety of other metalloenzymes (Fig 1A). These enzymes are characterized by an $HXXEHX_{74-76}E$ active site motif, a motif that could apply to G1L if the zinc-binding downstream residue is E120. E35, a highly conserved residue not predicted to participate in the catalytic activity of G1L, was also mutated in order to gain an understanding of the roles played by other charged residues that surround the active site. A study conducted by Kitada et al [23] demonstrated that a conserved E residue just upstream of the active site motif functioned as a necessary acidic residue since mutational analysis demonstrated a loss of enzyme activity upon the replacement of E47 with A but a partial restoration of activity when an aspartic acid (D) was substituted for E47. Each construct in this study was engineered with a flag epitope (DYKDDDDK) on the C-terminus for detection in immunoblot analysis (Fig. 1B) and was placed under the control of either the synthetic early-late promoter [31], as in the case of constructs used in transient expression assays, or the native G1L promoter, which is located in the region 229 basepairs upstream of the G1L initiating codon, for constructs employed in rescue assays.

Previous studies have demonstrated that during the VV replication cycle G1L undergoes an internal cleavage event [26]. At this point it remains unclear as to whether G1L participates in autoproteolysis or exists as a substrate of another viral or cellular proteinase. In an effort to get one step closer to answering this question, the fate of the G1L mutant plasmid library was analyzed in the context of a VV infection. BSC_{40} cells [1] were transfected with 1.5 μg of plasmid DNA containing either wild type G1L or one of the eight single-site mutants by way of a liposome-dependent transfection procedure. Four hours later, the transfection solution was removed and cells were infected with VV strain Western Reserve (WR) at a multiplicity of infection (MOI) of two. Twenty-four hours later, cells were harvested and extracts were subjected to immunoblot analysis using anti-flag antisera. Results indicate that in each case full length G1L is expressed at approximately 66 kDa as indicated by the arrow (Fig. 3, upper panel). However, a cleaved product is only observed for wild type G1L and G1L containing a mutation at E120. The mutant construct E35A also appears to undergo some degree of cleavage albeit to a much lesser extent (Fig. 3, lower panel). Taken together, these results suggest that alterations in the amino acid sequence of the putative active site of G1L as well as three of the four potential downstream zinc-binding residues renders the protein either unable to perform autocatalysis or unrecognizable as a substrate for

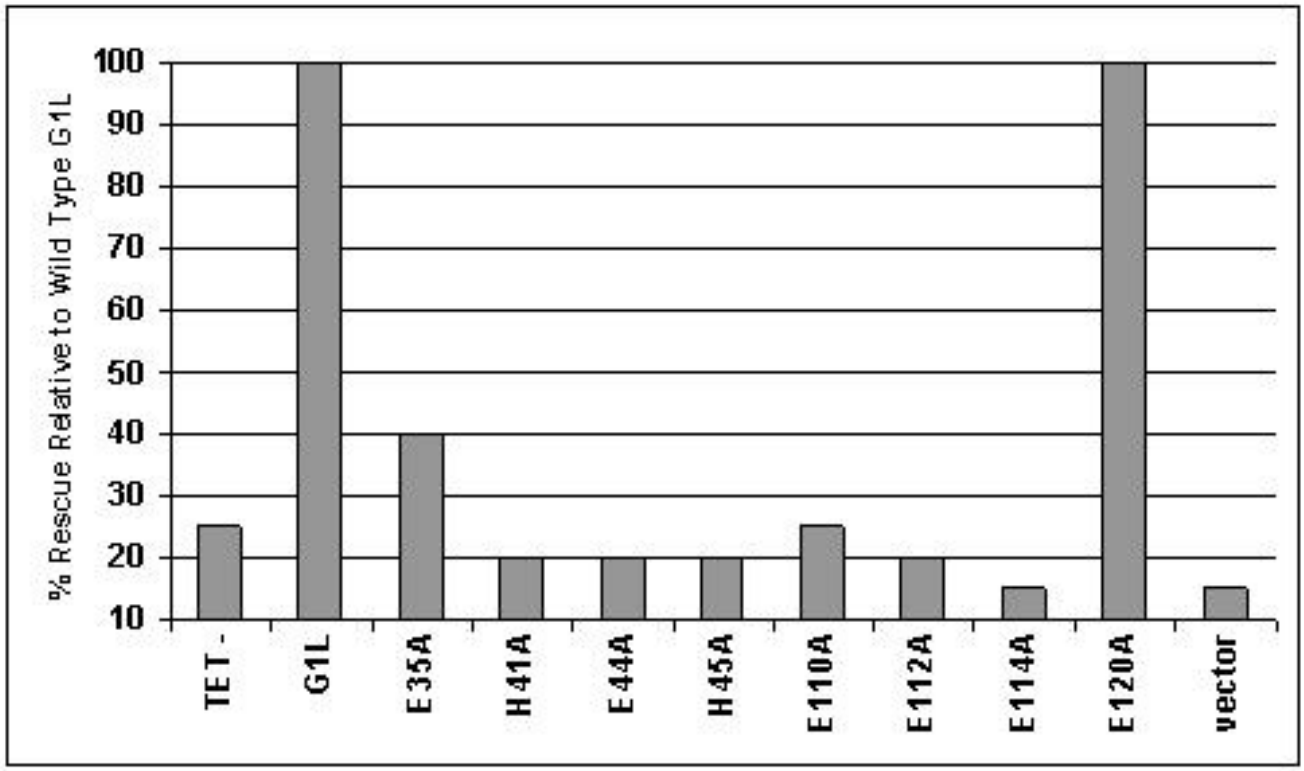


Figure 4
Mutational analysis of the putative catalytic and zinc-binding residues of G1L utilizing a *trans* complementation assay. TREx 293 cells were transfected with 1 μg plasmid DNA bearing either wild type G1L or one of the single-site mutants. Four hours later the transfection solution was removed and replaced with infection media containing vvtetO:G1L at an MOI of 0.1 Cells were harvested at twenty-four hours post infection, subjected to a series of rapid freeze/thaws and titered via plaque assay on BSC_{40} cells. Bars represent the percent rescue of each construct relative to what was achieved by transfection with the wild type G1L construct (G1L). Each transfection was carried out in the absence of TET (TET-).

another proteinase. Additionally, these results imply that E120 would not be the downstream residue involved in the coordination of a zinc ion.

To determine the role of these conserved residues in G1L activity, the mutant G1L constructs were evaluated for their ability to rescue viral replication in a *trans* complementation assay utilizing a tetracycline (TET)-dependent recombinant VV, vvtetO:G1L. The construction of vvtetO:G1L is described in detail in 27. Briefly, the TET operator was inserted directly upstream of the G1L open reading frame. When used in conjunction with a commercially available cell line expressing the TET repressor, expression can be controlled by either the presence or absence of TET within the culture media. In our assay, TREx 293 cells (Invitrogen, Carlsbad, CA) were transfected with 1 μg plasmid DNA bearing either wild type G1L or one of the single-site mutants. Four hours later the transfection solution was removed and replaced with infection media containing vvtetO:G1L at an MOI of 0.1 Cells were harvested at twenty-four hours post infection, subjected to a series of rapid freeze/thaws to release intracellular virus particles and titered by plaque assay on BSC_{40} cells. Figure 4 shows the average percent rescue obtained for each of the mutant constructs relative to wild type G1L and is the culmination of two independent experiments. With the exception of wild type G1L, the E120A mutant and, to a lesser extent the E35A mutant, none of the other mutants were capable of complimenting the conditional mutant virus grown in the absence of TET. These results are in accordance with what was observed in the transient processing assay and suggest that cleavage of G1L is a necessary event in order for VV to propagate efficiently. The fact that the E35A construct conveys only partial rescue correlates with the reduction in proteolysis observed by immunoblot.

With the recent identification of I7L as the VV core protein proteinase [11-13], the function of G1L within the viral replication cycle remains an enigma. Initially, the core proteinase was thought to be G1L due to discovery of an HXXEH inverted zinc-binding motif common to members of the M16 family of metalloproteinases (Fig. 1A). This motif is 100% conserved among poxviruses implying it is the enzyme active site. Additional sequence analysis and structural modeling further support the hypothesis that G1L may be the first example of a virally encoded metalloenzyme, although, G1L has not yet been shown to coordinate a zinc ion. To date, G1L expression is known to be an essential part of a VV infection. It has also been shown to undergo proteolytic processing, which appears to be essential for activity.

In this manuscript we report on the analysis of eight highly conserved residues within VV G1L including the three conserved residues located within the HXXEH catalytic motif as well as four downstream E residues, one of which is believed to be the essential downstream residue involved in the coordination of a zinc ion (Fig. 2). A library of C-terminally flag-tagged G1L constructs containing single point mutations to each residue was generated (Fig. 1B). Transient expression assays allowed us to monitor the processing of each construct through the detection of a C-terminal cleavage product. Cleavage was observed for wild type G1L as well as the construct containing a mutation to E120 (Fig. 3), however G1L appeared unable to tolerate mutations in E110, E112 or E114. Further, mutations in H41, E44 or H45 also inhibited G1L processing. This may be indicative of either the loss of a zinc ion, which would render a metalloproteinase inactive or the destabilization of protein folding resulting in the inability of G1L to be recognized as a substrate. Interestingly, mutation of E35, a highly conserved residue not predicted to participate in zinc-binding or substrate association, appears to affect G1L activity as well, although these effects were less dramatic than what was observed for the other mutant constructs as evidenced by the presence of a very faint C-terminal product. The lack of a robust cleavage reaction suggests the presence of an alanine at this position may destabilize the protein structure enough to make processing inefficient. Furthermore, rescue of a conditionally lethal mutant virus grown under non-permissive conditions was only observed with the E120A mutant (Fig. 4), which produced a phenotype nearly identical to the phenotype observed for wild type G1L. The E35A mutant also demonstrated the ability to rescue; however, rescue was markedly diminished relative to wild type G1L. In no other case did rescue reach above 25% of wild type.

These results suggest that G1L may fit into the paradigm of what is observed for other metalloproteinases and proteolytic enzymes in general in that G1L may initially be translated as an inactive zymogen, which is then activated upon proteolysis. In the absence of G1L expression, arrest in replication occurs subsequent to the activities of I7L, but prior to complete core condensation [27] suggesting the involvement of G1L in a proteolytic cascade. Of course these data do not rule out the possibility that the processing observed is simply an artifact of over-expression similar to what is observed for membrane-type-1 MMP, which may undergo autoproteolysis to an inactive form as a means of negative regulation in response to conditions of over-expression [32]. This scenario is unlikely, however, since the C-terminal region of G1L was observed via silver stain in the context of a conditional mutant VV infection [26].

The role of both the full-length and cleaved products of VV G1L continue to be investigated as does the ability of G1L to coordinate a zinc ion. If G1L is recognized as a bona-fide metalloproteinase, it will be of interest to determine if it acts alone or has a requirement for an additional subunit, much like what is observed for other MPPs. Further, once an appropriate expression system is established, analysis via mass spectroscopy may be used to determine exactly where G1L cleavage occurs, which may in turn aid in the identification of potential G1L substrates.

Competing interests

The author(s) declare that there are no competing interests.

Authors' contributions

KMH conducted the experiments and wrote the manuscript. CMB constructed the conditional lethal virus, assisted with the creation of the figures and edited the manuscript. DEH conceived the study, coordinated the research efforts and edited the manuscript. All authors read and approved of the final manuscript.

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REVIEW

Vaccinia virus proteolysis—a review

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SUMMARY

It is well known that viruses, as obligate intracellular parasites, must use their hosts' metabolic machinery in order to replicate their genomes and form infectious progeny virions. What is less well known are the details of how viruses make sure that once all the necessary proteins are made, that they assume the correct configuration at the proper time in order to catalyse the efficient assembly of infectious virions. One of the methods employed by viruses to regulate this process is the proteolytic cleavage of viral proteins. Over the past several decades, studies in numerous laboratories have demonstrated that morphogenic proteolysis plays a major and essential role during the assembly and maturation of infectious poxvirus virions. In this review we describe the history of vaccinia virus proteolysis as a prototypic viral system. Copyright © 2006 John Wiley & Sons, Ltd.

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GENERAL OVERVIEW OF VIRAL PROTEOLYSIS

The term 'limited proteolysis' was first introduced by Linderstrom-Lang and Ottesen to describe reactions in which the peptide bonds in a polypeptide are selectively hydrolysed, as opposed to protein degradation which involves extensive cleavage of the peptide bonds in the substrate [1]. The enzymes required for the peptide bond cleavage are named proteases which are divided into peptidases and proteinases [2]. Peptidases are exopeptidases which hydrolyse single amino acids from the amino-terminus or the carboxy-terminus of a peptide chain. In contrast, the proteinases (also called proteolytic enzymes or endopeptidases) are capable of selectively recognising and cleaving specific peptide bonds within substrates.

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Abbreviations used
VV, vaccinia virus; IMV, intracellular mature virus; IEV, intracellular enveloped virus; EEV, extracellular enveloped virus; CEV, cell-associated enveloped virus; CPV, cowpox virus; TK, thymidine kinase; vCPP, vaccinia virus core protein proteinase; EMC, encephalomyocarditis virus

Proteinases are further subdivided into four classes based on the identity of their catalytic amino acid residues, whose relative three-dimensional positions are conserved within a group, and the mechanism of catalysis [2]. The four types of proteinases are: serine, cysteine (thiol), aspartic (acid), and metallo. Serine proteinases possess a catalytic triad of aspartic acid, histidine and serine residues, and appear to be the most common and widespread type of proteinase. The serine residue is usually the amino acid that acts as a nucloephile during the reaction by donating an electron to the carbon of the peptide bond to be cleaved. A proton is then donated to the leaving amino group by the histidine residue. The serine is hydrolysed, the product released, and the active site is regenerated [1]. Cysteine proteinases maintain a catalytic diad composed of cysteine and histidine residues in close proximity. In some cases there is a catalytic triad with the addition of an aspartic acid residue which helps in the stability of the active site. The mechanism of action is similar to that of serine proteinases except that the nucleophile is a thiolate ion instead of a hydroxyl group. The sulphhydryl group of the cysteine residue acts as the nucleophile to initiate attack on the carbonyl carbon of the peptide bond to be cleaved. The imidazol ring of the histidine residue removes a proton from the sulphhydryl making it more nucleophilic. Catalysis proceeds through the formation of a

covalent intermediate. Unlike serine and cysteine proteinases, aspartic and metalloproteinases do not appear to form a covalent enzyme–substrate intermediate [3]. Aspartic proteinases have a catalytic diad of two aspartic acid residues. Catalytic activity occurs as two molecules join together bringing the aspartic acid residues into close proximity of each other. Acid-base catalysis from an activated water molecule leads to the formation of a non-covalent tetrahedral intermediate. For the metalloproteinases, a divalent cation (usually Zn^{2+}) is required together with essential histidine and glutamic acid residues for catalysis. As with aspartic proteinases, the catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the zinc-bound water molecule attacks the carbonyl group of the scissile bond.

Proteinases can be thought of in their most basic form as having a catalytic site, as described above and a substrate binding pocket [4]. The two sites are usually in close proximity. Generally, proteinases are composed of two globular domains, with amino acids involved in catalysis being contributed by each half of the substrate-binding crevice. For most serine, cysteine and aspartic proteinases, the two globular domains are found within the same polypeptide. However in the case of the retroviral proteinases [5], a dimer complex is employed to bring together two individual catalytic centres to form the crevice. Although nearly all substrate-binding crevices achieve a similar three-dimensional structure with respect to the catalytic amino acids for each class of proteinase, the structural conservation does not extend to the substrate binding pocket, which distinguishes a given proteinase from all others. It is this substrate binding region which confers specificity to the proteinase.

It is generally accepted that for the hydrolysis of a specific peptide bond to occur, two requirements must be met. First, the susceptible peptide bonds need to be defined by the nearby amino acid residues with specific side chains which are required for the primary and secondary specificity. According to Polgar [2], the primary specificity has a qualitative feature which targets the selection of the scissile bond, and the secondary specificity conveys a quantitative feature by facilitating the cleavage of the selected bond. Second, the susceptible bond is usually displayed adjacent to the surface of the substrate in a flexible region accessible to the proteinase, and the susceptible peptide must be presented in a three-dimensional conformation which fits the active site pocket of the proteinase. This is referred to as conformational specificity [6].

Many types of post-translational modifications such as phosphorylation, glycosylation, and acylation are required for the acquisition and regulation of protein properties such as enzyme activity, protein–protein interactions and intracellular localisation. Likewise, limited proteolysis is often used to regulate protein activation or assembly by causing changes in the tertiary structure which bring distant functional amino acid residues together. Interestingly, the free energy required for the reconstruction of the hydrolysed peptide bond is high and no biological mechanisms for repairing the broken peptide bond have yet been identified. Thus the changes introduced into substrates by proteolytic cleavage are essentially irreversible. This combination of cleavage specificity and reaction irreversibility have resulted in the common utilisation of the proteolytic processing reaction as a unidirectional mechanism for a wide variety of biological processes including food digestion, signal peptide cleavage, signal transduction, peptide hormone/growth factor production, blood clotting, complement pathway cascade, pathogen elimination, cell migration and reproduction [7–9].

For many plant and animal viruses, a successful infection is dependent on proteolytic processing at one or more stages. In fact, it is the exceptional virus that does not require proteolytic processing during its replication cycle [3]. The required proteolytic enzymes can be provided by either the host cell, the infecting virus, or both. Proteinases provided by the host cell generally contribute to the processing of membrane or envelope proteins that are trafficking through the secretory compartment of the cell. It is within these secretory compartments that viral envelope proteins undergo maturation by cleavage of signal peptides (in addition to acylation and glycosylation), such as the E1 and E2 glycoproteins of Sindbis virus [10]. On the other hand, the proteinases which are responsible for the proteolytic processing of viral proteins are usually encoded by the viruses themselves.

Proteolytic cleavage of viral polypeptides have been categorised as 'formative' or 'morphogenic' proteolysis, depending on the function the reaction serves during the replicative cycle [11].

Formative proteolysis refers to the processing of viral polyproteins into structural and non-structural protein products. A number of viral formative cleavage proteinases have been identified and are encoded by animal viruses such as picornaviruses, flaviviruses, alphaviruses, retroviruses and coronaviruses [12]. Formative proteolysis provides a mechanism for viruses, such as retroviruses and positive-strand RNA viruses, to utilise a single RNA template for the expression of several viral proteins from a large polyprotein precursor. Morphogenic proteolysis refers to the cleavage of viral structural proteins assembled in previrions during virion maturation. Morphogenic cleavage occurs in conjunction with virion assembly and is often required for the acquisition of infectivity of both DNA and RNA viruses such as picornaviruses, alphaviruses, retroviruses, adenoviruses, and bacteriophage T4 [13]. Although less is known about morphogenic proteolysis, several different functions have been proposed for this process, including: facilitation of correct genomic RNA dimerisation in assembling retroviral particles [14]; unidirectional packaging of bacteriophage T4 DNA [15]; completion of the infectious poliovirus virion in a flexible configuration [16]; and, promotion of proper disassembly of adenovirus particles during the initiation of infection [17].

In addition to formative or morphogenic proteolysis, cleavage reactions can be further described as being either *cis* or *trans*. In *cis* cleavage events one precursor protein contains both the cleavage site and the proteinase activity, which is autocatalytic. In *trans* cleavage events, one protein contains the proteolytic activity and a second contains the substrate cleavage site.

In the most basic case, proteinases are synthesised in their active form, as has been shown through TNT reactions where the enzyme and substrate can be synthesised and activity demonstrated [18–20]. Some proteinases need to be cleaved from a precursor protein in order to become activated while other proteinases require cofactors for catalysis to occur, such as in the adenovirus system where the protease requires both DNA and a specific peptide as cofactors [21,22].

Figure 1 depicts the general requirements for proteolysis including the location of the scissile bond in the protein to be cleaved, the difference between *cis* and *trans* cleavage, and the difference between formative versus morphogenic cleavage.

Regardless of the type of proteolytic maturation employed, it is essential that the activity of the viral proteinase be properly regulated to ensure the efficient production of infectious progeny virions. In general, within biological systems, regulation of proteinases is achieved in several ways including differential compartmentalisation of the enzymes and substrate, presence of specific inhibitors and/or activators, and the proteolytic activation of zymogens. Viruses have adopted similar strategies. For example in the retroviruses, it has been proposed that the acidic extracellular environment triggers the morphogenic cleavage of structural proteins by displacing a portion of the *gag-pol* polyprotein which prevents the active site of the proteinase

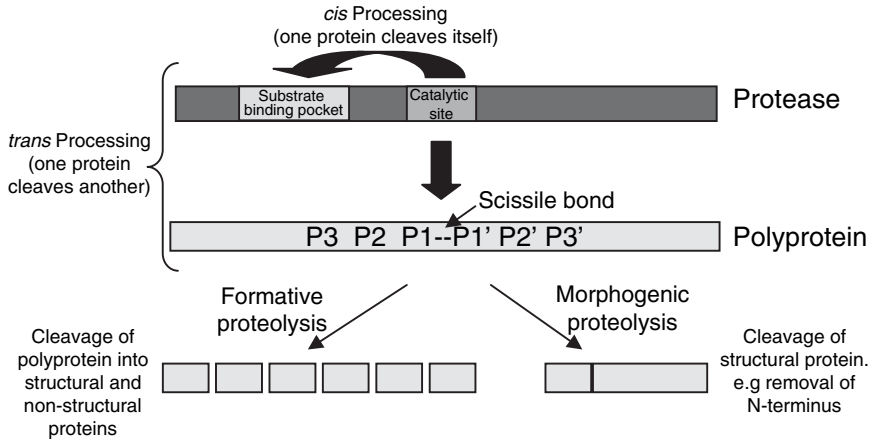

Figure 1. Depiction of the general requirements for proteolysis

from interacting with its substrate while within the cell [11]. In the case of adenoviruses, it appears that a disulphide-linked peptide produced from the pVI structural protein during the latter stages of replication is required for the activation of the viral proteinase and subsequent virus maturation [21]. Finally, perhaps the most elegant example of regulating viral proteinase activity is provided by the core protein of Sindbis virus which undergoes autoproteolysis to become inactive after assembly of the nucleocapsid. Inactivation of the proteinase activity is accomplished by locating the carboxy terminal region of the protein into the catalytic pocket in concert with the proteolytic cleavage event [23].

VV REPLICATION CYCLE AND POSTTRANSLATIONAL MODIFICATION OF VIRAL GENE PRODUCTS

Given the importance of limited proteolysis as a means to regulate gene expression in biological systems, and the extent and diversity of ways that even simple viral systems apparently employ this regulatory mechanism, it is of interest to consider if and how a complex virus such as vaccinia virus might incorporate this process into its replicative cycle. Vaccinia virus (VV) is the prototype of the *Poxviridae*, a family of DNA viruses distinguished by their unique morphology and cytoplasmic site of replication [24]. The 191 kbp VV DNA genome encodes at least 263 gene products whose expression is regulated in a temporal fashion during the viral replicative cycle that begins with entry of the virus into the host cell and terminates with the assembly of complex macromolecular structures to form an infectious particle (Figure 2). Unlike many other viruses, VV produces a multiplicity of virion forms, all of which appear to be infectious. Although the molecular details of poxvirus assembly and differentiation remain controversial, the most widely accepted scenario of events which transpire is as follows. After (or concurrent with) viral DNA replication, assemblages of progeny DNA molecules, virion enzymes and structural proteins coalesce to form pre-virion particles [25]. These particles then acquire membranes, whether one or two membranes still remains controversial, to become infectious intracellular mature virus (IMV). There are two hypotheses about the origin of the membranes; one holds that the membrane is synthesised *de novo*, while the other hypothesis holds that the membrane is acquired by budding though the intermediate compartment (between the endoplasmic reticulum and the Golgi). A portion of the IMV then become enveloped by additional membranes derived from the trans-Golgi network to form intracellular enveloped virus (IEV). Following migration to the cell surface the outermost IEV membrane fuses with the plasma membrane to give rise to extracellular enveloped virus (EV) [26]. The EV can either remain associated with the cell (cell-associated enveloped virus, CEV) or be released into the external medium as extracellular enveloped virus (EEV) [27]. Some poxviruses, such as cowpox virus (CPV), produce yet another virion form. In CPV-infected cells, large inclusion bodies are produced which are composed primarily of a single 160 kDa viral protein [28]. Within these A-type inclusions are occluded (and infectious) virions. All of the proteolytic reactions that have been characterised for VV to date are involved in IMV formation

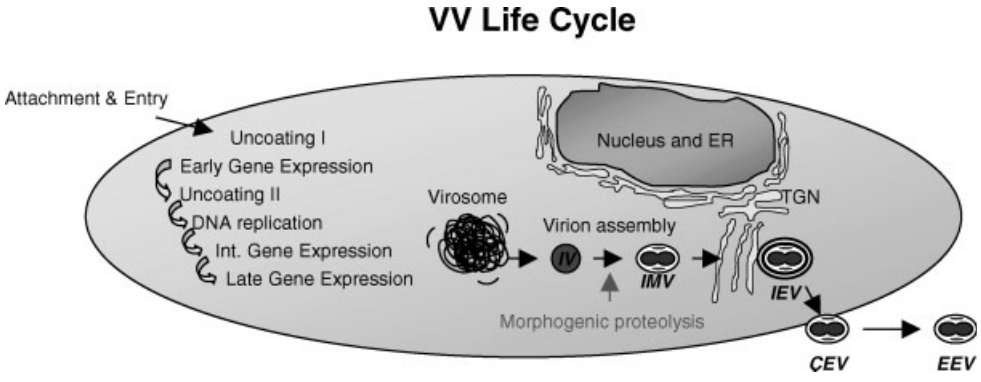

Figure 2. Vaccinia virus replicative cycle

and not the subsequent maturation of IMV to IEV, EV or CEV.

It is well known that viruses, as obligate intracellular parasites, must use (and in some cases redirect) their hosts' metabolic pathways in order to replicate their genomes and synthesise the constituents needed to form progeny virions. What is perhaps less well appreciated are the logistical problems encountered by viruses during the replicative process. Once viral proteins have been synthesised, how does the virus ensure that these proteins assume active configurations (at the proper time), interact with the correct protein partners and find their way to specific intracellular locations in quantities sufficient to catalyse the efficient assembly of infectious virions? One of the major ways that viruses solve these problems is by taking advantage of protein modification pathways which are normally used to assist with intracellular trafficking of cellular proteins [29–33].

Thus, considering the large number of viral encoded proteins, the multiplicity of VV virion forms and the number of distinct intracellular sites used during the viral assembly and morphogenesis process, one would predict that VV might utilise a number of cellular protein modification and targeting pathways to regulate these complex processes. Indeed, it has been demonstrated that during the course of viral replication, VV proteins are matured by a number of post-translational modifications including myristylation [29], acylation [30], phosphorylation [31], glycosylation [32], ADP-ribosylation [34], and proteolytic processing [33]. Although the details about what role limited proteolytic reactions might play during VV replication were not available when our studies were initiated in 1990, the information that was available in the literature suggested that both formative and morphogenic cleavage pathways might be employed. For example, both the VV growth factor (VGF) [35, 36] and haemagglutinin (HA) proteins [37] appeared to have signal peptides removed via formative proteolysis during their transit through the endoplasmic reticulum and transport to the plasma membrane. Likewise, three of the major structural proteins found in the mature VV virion core, 4a, 4b and 25K, were known to be produced from higher molecular weight precursors at late times during infection, making them candidates for morphogenic cleavages [24, 38]. It was this latter question, namely the nature of the processing reaction by which the major VV core proteins are matured, that the experiments conducted in our laboratory over the last decade have sought to address.

VV PROTEOLYSIS–WHAT WAS KNOWN PRIOR TO 1990

The genes expressed at late times during a VV infection (i.e. those expressed after the initiation of viral DNA synthesis) include most of the structural proteins required for the assembly of progeny virions. The first indication that some of the VV structural proteins might be subject to proteolytic processing occurred when Holowczak and Joklik noted differences in the apparent molecular weights of radioactively-labelled proteins present in VV-infected cells when compared to those found in purified virions [39]. Subsequently, pulse-labelling of VV-infected cells was used to demonstrate that a large precursor protein could be chased into a smaller polypeptide, with concomitant disappearance of the larger sized protein [40]. This conversion could be specifically inhibited by rifampicin with no apparent effect on the synthesis of the precursor. The precursor protein was designated as P4a and the proteolytically processed product called 4a [41]. Additional pulse-chase experiments revealed that several other VV structural polypeptides, in addition to P4a, were apparently cleaved during the late phases of the VV replication cycle [42]. These proteolytically processed proteins, referred to using the Sarov and Joklik designation of virion proteins [43], included 4a, 4b, 8 (referred to as 25K here), 9 and 10. This may in fact represent an underestimate of the number of VV late proteins which are subject to proteolysis, as Pennington [44] reported eleven late proteins disappeared during pulse-chase experiments and seven new proteins appeared. The VV core proteins 4a, 4b and VP8 (25K) are the most abundant proteins in the VV particle, together constituting about 33% of the mass of the virion. Tryptic peptide mapping and immunological reagents have been used to establish the relationships between the P4a, P4b and P25K precursors and their processed products 4a, 4b and 25K [38,45]. Location of the three loci encoding these genes has been mapped and the nucleotide sequence of their open reading frames determined [46, 47]. With the completion of the sequence of the entire genome of the Copenhagen strain of VV, the genes encoding the P4a, P4b and P25K precursors

received the designations A10L, A3L and L4R, respectively [48]. The proteolytic processing of VV structural proteins appears to be essential for the formation of infectious progeny virions. This conclusion stems from the observation that there are a variety of different drug treatments (e.g. rifampicin and α-amanitin) or conditional-lethal mutations in the genome, which apparently affect proteolysis (and particle maturation) without affecting overall protein synthesis [33].

IDENTIFICATION OF THE AG(X) CLEAVAGE MOTIF

By pulse-labelling VV-infected cells after the onset of viral DNA synthesis and analysing the extracts by SDS:polyacrylamide gel electrophoresis and autoradiography, one observes three major radiolabelled protein species with apparent molecular weights of 97 kDa (P4a), 65 kDa (P4b) and 28 kDa (P25K). Upon addition of media containing an excess of unlabelled amino acids and with continued incubation of the infected cells, these three proteins are chased into 62 kDa (4a), 60 kDa (4b) and 25 kDa (25K) species that co-migrate with the three major virion core proteins. Interestingly, unlike many proteolytic reactions, the conversion process is not rapid with a delay of about 30–45 min observed between the time of precursor synthesis and the appearance of cleavage product. Furthermore, proteolysis of the VV core protein precursors appears to require ongoing *de novo* protein synthesis as addition of cycloheximide at the time of the chase completely inhibits product formation. Both of these observations are in agreement with the hypothesis that cleavage occurs within the context of an assembling virion, a process which requires both time and continual protein synthesis, and can be referred to as 'contextual processing'.

To determine the sites at which the VV core protein precursors were being cleaved, mature 4a, 4b and 25K proteins were isolated, purified and subjected to N-terminal microsequence analysis. The derived sequence was then compared against the predicted amino acid sequences of the P4a, P4b, and P25K ORF's. The amino termini of the 25K and 4b proteins were identified as residue 33 of P25K and residue 62 of P4b, respectively [49]. The predicted decrease in molecular weight due to the loss of 32 and 61 amino acids from the N-terminus of P25K or P4b, respectively, corresponded well with the shift in migration observed in gels following proteolytic processing. Comparison of the predicted amino acid sequences within the P4b and P25K precursors which surround the derived N-termini of the mature 4b and 25K proteins revealed the presence of an identical motif. The N-termini of both cleavage products were found within a conserved Ala-Gly-Ala (AGA) tripeptide, with the predicted cleavage site occurring at the Gly-Ala scissile bond. The importance of the AGA motif as a cleavage site determinant was suggested by two lines of evidence. First, there were no other obvious sequence elements conserved upstream or downstream of the putative P4b and P25K cleavage sites. Second, although the fowlpox virus (FPV) 4b and 25K core protein homologues only share 52 and 33% identity, respectively with the VV proteins [50], both FPV precursors contained an AGA motif at exactly the same location as the VV core protein precursors. Interestingly, although the 4a protein was subjected to similar analyses, no microsequence data was obtained, suggesting that its N-terminus was blocked.

In view of the failure to obtain amino acid sequence from purified 4a protein, it was of interest to note that the predicted amino acid sequence of the P4a precursor did not contain the conserved AGA tripeptide motif utilised in the processing of P4b and P25K. This raised the possibilities that the P4a protein was processed by a different pathway, or that P4a was processed by the same pathway but at sites that were less efficiently cleaved. Support for the latter hypothesis was drawn from the observation that cleavage of the P4a precursor seems to proceed at a slower rate than that of the P4b and P25K precursors *in vivo* [51]. To determine the cleavage site of P4a, immunological reagents which were specific for subregions of the P4a precursor were generated and used in concert with a variety of peptide mapping and protein sequencing procedures. The results obtained demonstrated that the P4a precursor was cleaved at two locations, the AGS site between residues 613 to 615 and the AGT site between residues 697 and 698 [51]. Both the large N-terminal 4a protein (residues 1 to 614) and the C-terminal 23 kDa protein (residues 698 to 891) become major virion core constituents. The location and fate of the small internal peptide predicted to be released (residues 615–697) is not known. However, using transient expression procedures, mutant P4a proteins were

produced in which either the AGS or AGT sites had been genetically inactivated. Under these conditions it was possible to demonstrate the existence of the 4a-9K or 9K-23K proteins *in vitro*, suggesting that the internal 9 kDa sequence is not inherently unstable. Furthermore, the ability to isolate and microsequence both end products from cleavage at the AGS and AGT sites also strongly suggested that processing was occurring via a single endoproteolytic cleavage event. Although the internal AGS and AGT sites of the P4a precursor were cleaved, two independent lines of peptide mapping data clearly demonstrated that the AGN site at residues 94 to 96 was not processed. Thus, these results suggest that processing of all three major core protein precursors is likely to be coordinately lined and catalysed by the same viral proteinase during viral assembly, with the endoproteolytic cleavage occurring at an internal AGX motif (where X can be A, S, or T, but not N).

CONTEXTUAL PROCESSING

All three of the VV core proteins that were known to be proteolytically processed are cut at AGX motifs. It was therefore of interest to ask within the context of the entire VV genome, how many times an AGX tripeptide is predicted to be present in a VV protein, and to determine the frequency at which the motif is utilised as a cleavage site. Using the complete nucleotide sequence of the VV (Copenhagen) genome [48], the predicted amino acid sequence of each ORF was determined and compiled into a single database. A search of this database for the AGX tripeptide revealed it occurred 82 times among the 198 major ORF's, which is substantially less frequent than the 204 sites expected if AGX occurred randomly. Of these 82 sites, 18 resembled sites that had previously been shown to be actively cleaved, namely AGA of P25K and P4b, and AGT and AGS of P4a.

To explore whether some or all of these AGX motifs serve as cleavage sites during VV replication, we focused our attention on the AGA tripeptide as a test case. The AGA motif occurs seven times. The proteins containing the AGA motif are: P4b and P25K core protein precursors (both of which are cleaved), the A12L and A17L gene products, the palmitylated P37 protein encoded by the F13L ORF which is found in the outer membrane surrounding EEV particles, the VV DNA polymerase (DNAP) encoded by the E9L ORF, and host range (HR) protein encoded by the K1L gene. To determine if these proteins were processed during the course of a VV infection, monospecific antisera were produced or obtained (αDNAP from P. Traktman and αP37K from R. Wittek) for each individual gene product and used in concert with pulse-chase radiolabelling and immunoprecipitation procedures. The results obtained clearly indicated that the DNAP, P37K and HR proteins were *not* proteolytically processed. In contrast, the gene products of the A17L and A12L ORF's were synthesised as 23 kDa and 24 kDa precursors which were processed to 21 kDa and 17 kDa products. The processing reaction was inhibited by rifampicin and the processed products were matured by the same pathway as the P4b and P25K precursors [52]. These results allowed us to propose several rules governing the morphogenic cleavages in VV.

To be processed, a precursor protein must: (1) Contain an AGX motif, (2) Be expressed at late times during infection (DNAP is an early protein, HR is an early-intermediate protein); (3) Be destined for incorporation into the virion core (P37 is a late protein but is located in the membrane not the core). Even with these stringencies, given the number of AGX occurrences plus the large number of proteins found in the complex VV virion, this suggests that as many as 40 to 50 viral proteins may be subject to morphogenic cleavages during virion assembly. Mercer and Traktman [53] have recently shown that the vaccinia virus G7 protein, which plays a role in virion assembly, undergoes cleavage at two AGX sites, AGF (aa182–184) and AGL (aa 237–239), with the processing being important for virus viability. Figure 3 highlights the AGX cleavage sites in several vaccinia virus proteins that are known to undergo cleavage.

VV CORE PROTEIN PROCESSING IS A MORPHOGENIC CLEAVAGE PATHWAY

As an approach to test whether VV core protein proteolysis serves a formative or morphogenic function in the virus life cycle, a method of sucrose log gradient fractionation was developed which allowed the separation and purification of radiollabelled immature and mature VV particles from four distinct peaks based on varying rates of sedimentation [54]. Slower sedimenting fractions were

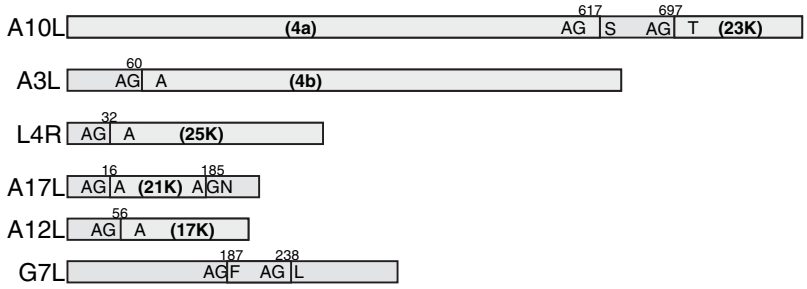

Figure 3. AGX cleavage sites in vaccinia virus proteins

found to move faster through the gradient over time using pulse-chase procedures, indicating precursor-product relationships due to proteolytic maturation. The slowest sedimenting product was found to contain predominantly uncleaved core protein precursors while the fastest moving products were composed almost entirely of the mature core proteins 4a, 4b, 25K and 23K. These results showed that several distinct and separable forms of VV previrions exist, that VV core protein precursor s are associated with previrions prior to cleavage, and that maturation of the core proteins is coordinately linked to the conversion from non-infectious previrions to infectious viral particles. This provides strong support for the hypothesis that VV core protein precursor maturation serves a morphogenic function [55].

In another set of experiments designed to monitor the intracellular and intraviral localisation of the VV core proteins during infection, a collection of monospecific polyclonal antisera were produced that recognised individual core protein precursors and/or their cleaved products. Use of these sera in indirect immunofluorescence analyses of VV-infected cells demonstrated that the VV core protein precursors were not distributed throughout the cytoplasm of infected cells. Rather, the core protein precursors were localised almost exclusively to the 'virosomes or virus factories' where the progeny virions were being assembled. At late times during infection, punctate staining was also evident throughout the cytoplasm which we believed to be individual virus particles. This hypothesis was confirmed by immunoelectron microscopy. The IEM studies also demonstrated that VV core protein precursors were associated with immature VV virions and that as the virions progressed through the maturation cycle the newly-processed core proteins remained associated with the condensing core. [55]

The results of the experiment reported above indicate that the precursor form of the VV core proteins is required for proper localisation to the virosome and assembling previrion. This raises the question of what feature of the core protein precursors is responsible for this behaviour, as not all proteins present in a VV-infected cell are packaged. One can propose that it is the overall structure of the core proteins, the protein partners with whom they interact, or the presence of a specific targeting signal that provides this property. At least in the case of P4b and P25K, one obvious candidate for a potential targeting signal is the N-terminal leader peptide that is removed by proteolysis during viral assembly. To test this hypothesis, a mutant P25K gene was constructed in which the sequence encoding the leader (the 31 N-terminal amino acids) was deleted. The (Δ31)P25K protein was expressed in the context of a VV-infected cell via transient expression. This protein, which should be functionally equivalent to mature 25K core protein, was not packaged into virions. To determine if the entire leader of the core protein precursor was required to provide this phenotype, a set of deletions in the 61 amino acid leader of the P4b protein were constructed. Deletion of 15, 30, or 44 amino acids had no effect on P4b processing, indicating that the essential information was proximal to the cleavage site. Proof that it was the sequence or structure of the leader which imparted this property was provided by fusing the N-terminal 30 amino acids of the viral thymidine kinase (TK), a soluble early protein, to the 25K protein in a manner which reconstituted the AGA site. This TK:25K fusion protein was neither packaged nor processed. Interestingly, the leaders of the core proteins appeared to be functionally interchangeable as two swap mutants were generated by making P4b leader:25K and P25K leader:4b chimeras. In both cases, the proteins could

be packaged and processed. This result argues against the overall tertiary structure of the core protein precursors being the primary localisation determinant as the fusion proteins would surely have disrupted structural features. Taken together, these data suggest that the amino terminal peptides of the VV core proteins are to some extent interchangeable and that the residues immediately proximal to the AGA site appear to contribute most directly to the correct intracellular and intraviral localisation. [56]

CHARACTERISATION OF THE CIS SIGNALS RESPONSIBLE FOR P25K CLEAVAGE

Based on precedents in the viral proteinase literature, a number of different approaches to developing an assay system to study the proteolytic processing of VV core proteins were attempted. These included: (i) *in vitro* cleavage assays mixing VV core protein precursors isolated from cells infected with cleavage-deficient temperature-sensitive VV mutants together with extracts from wild-type VV-infected or uninfected cells; (ii) mixing solubilised VV virions with VV core protein precursors made *in vivo* or *in vitro*; (iii) co-translation of core protein precursor mRNA with mixtures of cellular and/or viral mRNA in rabbit reticulocyte lysates, and (iv) transient expression assays using the hybrid T7/VV system [57] to express various reporter gene constructs containing putative VV core protein cleavage sites. Without exception, no cleavage of the test substrate was observed with any of these systems. This led, in part, to the working hypothesis that proteolytic maturation of VV core proteins is contextual, linked directly to virion assembly. The predictions of this hypothesis are that for a VV protein to be cleaved at the AGX motif it must be synthesised late in infection, packaged into the assembling virion and it needs to be associated with the VV core. Any perturbation of the kinetics of synthesis, intracellular targeting or structure of a VV core protein precursor might be expected to abrogate processing.

To test this hypothesis a trans-processing assay was developed [16] to follow VV core protein proteolytic maturation by tagging the P25K VV core protein precursor at the C-terminus with an octapeptide epitope, FLAG [58] (Figure 4). By using transient expression assays in cells co-infected with VV, the proteolysis of the P25K:FLAG fusion gene product could be monitored by immunoblotting and immunoprecipitation procedures using antisera specific for the FLAG epitope or the 25K protein. In tissue culture, the P25K:FLAG precursor was cleaved to smaller products and the precursor-product relationships were established by pulse-chase labelling protocols. The cleavage of the P25K:FLAG precursor was believed to be using the same pathway as authentic VV core protein precursors due to the inhibition of processing by rifampicin. The 25K:FLAG product was found to be associated with mature virions, agreeing with the hypothesis that cleavage occurs in conjunction with virion assembly. Site-directed mutagenic replacement of the AGA site within the

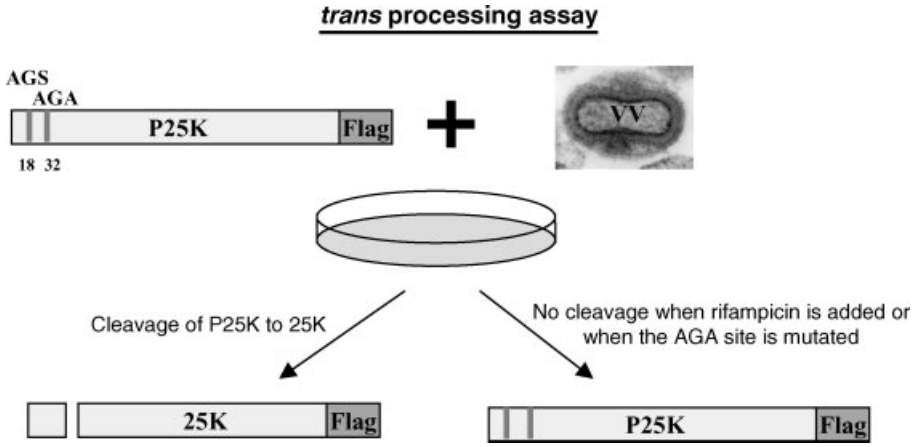

Figure 4. ***trans*** **processing assay. Cells are infected with vaccinia virus and transfected with plasmid borne P25K with a C-terminal Flag tag. Cleavage is monitored by immunoblotting**

P25K:FLAG precursor with the tripeptide IDI blocked cleavage at the mutated site, indicating that the AGA site was used and recognised independently, and that bonafide proteolysis was occurring. [16]

Although the central importance of the AGX motif in P25K processing is suggested by the failure of the IDI mutant to cleave, there must be additional determinants of cleavage site selection. Not all proteins containing an AGX motif are proteolytically cleaved. One such example is the AGN site of P4a which is not cut, despite the fact that two other downstream AGX sites in the same precursor are. Likewise, as discussed above, the VV DNA polymerase, palmitylated 37K envelope protein and the HR proteins all have AGA motifs which are not cleaved. Therefore, the AGX motif appears to be essential but not sufficient for defining a specific cleavage site. Additional substrate determinants within the precursors must contribute to cleavage site selection. As an approach to identify some of these other determinants, we employed the trans-processing assay described above in concert with site-directed mutagenesis procedures. Specifically, using the P25K:FLAG gene as the template, more than 50 different substitution, insertion and deletion mutants were introduced in and around the AGA site. The phenotype of each individual mutant was analysed by transient expression and immunoblotting procedures [59].

The results are summarised as follows, using the nomenclature of Schechter and Berger [60] in which the positions at the amino- and carboxyl-proximal residues are indicated as P1, P2, etc., and P1′, P2′, etc., respectively. The residue occupancy of the P1′ position was exceedingly permissive with only a proline substitution blocking cleavage. In contrast, for cleavage to occur, the occupancy of the P1 and P2 sites was restricted to just a few amino acids. The presence of additional structural elements is suggested by the observation that insertion or deletion of sequences immediately adjacent (amino- or carboxyl terminal) to the AGA motif completely halted cleavage. [59]. While there is abundant information about the structural requirements surrounding core protein cleavage [49, 51, 55, 56, 59, 52, 61, 42] until recently the proteinase(s) responsible for carrying out these cleavage reactions has remained unknown.

G1L

As mentioned above, a large number of different approaches were attempted to reconstitute VV core protein proteolysis *in vitro*, and all were unsuccessful due to the contextual requirements of this reaction. Therefore, in order to identify the proteinase which is responsible for the cleavage of the VV core protein precursors a tissue culture based mapping procedure was employed, making use of a transcriptionally-controlled *trans*-processing assay. Transcription of VV genes is tightly controlled by a regulatory cascade mechanism [62]. All of the enzymes required for the synthesis and modification of early mRNA are packaged into the infectious virion. Following entry into the cell, early gene expression initiates viral DNA replication which leads to successive expression of intermediate and late viral genes. During a normal viral infection, it has been shown that newly replicated, naked, viral DNA serves as the template for the expression of the late transcription factor genes (A1L, A2L, and G8R) as well as the late genes themselves [63]. Recent studies have demonstrated that transcription from an exogenously supplied late promoter in infected cells, whose DNA replication has been blocked by AraC, can be rescued by co-transfecting plasmid copies of the late transcription factor genes [64]. This finding provided the basis for the development of the transcriptionally-controlled *trans*-processing assay using a plasmid copy of the P25K:FLAG ORF as the reporter gene [61].

Cells infected with VV in the presence of AraC to block late gene expression from the viral genome were transfected with full-length VV DNA, resulting in the expression and cleavage of VV core protein precursors. This indicated that both synthesis and processing could occur under these conditions, and that the proteinase responsible for cleavage was likely the product of a VV late gene. In order to separate substrate expression and proteolysis, VV-infected cells treated with AraC were simultaneously transfected with plasmid copies of the following: (i) late gene transcription factors; (ii) a test substrate; and (iii) a potential source of proteinase. As the test substrate, the P25K:FLAG fusion gene was used. The initial source of proteinase was a set of 6 overlapping cosmid clones of the VV genome supplied by B. Moss [65]. Individually, cosmids 3 and 21 were able to rescue processing of the P25K:FLAG protein at the AGS site

found at positions 16–18 within the N-terminal leader of the precursor. As these two cosmids overlapped in the HindIII-G region, a cloned copy of the VV HindIII G fragment was tested and also found to direct cleavage of the substrate at the AGS site [61].

Previously, we had used computer analyses to search the predicted amino acid sequences of all of the ORF's in the genomic sequence of VV (Copenhagen) for the presence of motifs that might suggest proteinase activity. The only 'hit' we had was within the G1L ORF, which contained a HXXEH sequence motif, which is a direct inversion of the active site consensus sequence present in metalloproteinases such as thermolysin [66]. G1L is predicted to encode a 68 kDa late protein that is highly conserved amongst the Orthopoxviridae. Although there are examples of viral proteins that require zinc for activity, such as the rubella virus protease [67] and the hepatitis C virus NS5A replicase [68], to date there are no known true viral metalloproteases. Despite this, as well as the observation that the motif was backwards in VV, we nevertheless cloned the G1L ORF, expressed it *in vitro* and tested if for the ability to cleave VV core protein precursors. The plasmid borne G1L was capable of cleaving P25K:FLAG at the AGS, but not AGA site and was unable to cleave transiently expressed P4a or P4b. Mutation of either the conserved HXXEX motif of the G1L ORF or of the AGS site in P25K abrogated cleavage [61]. This implicated G1L as a potential VV metalloproteinase, but not the proteinase responsible for cleavage of each of the major core protein precursors.

While the exact biological function of VV G1L remains unknown, two different recombinant viruses regulating the expression of G1L have shown that G1L is essential for viral morphogenesis [69, 70]. However, in the absence of inducer, no obvious effects on the processing of the major core protein precursors is observed. An electron microscopic examination of the virus in the absence of G1L expression revealed that immature viral particles are formed and there is an initiation of core condensation but the viral particles lack the characteristic oval shape and complete core condensation characteristic of mature viral particles. These results would suggest that G1L activity is likely required at a stage after core protein processing and that more than one VV proteinase is required to produce mature virus.

VACCINIA VIRUS CORE PROTEIN PROTEINASE (vCPP)

As an approach to determining what type of proteinase might be the vaccinia virus core protein proteinase (vCPP), a collection of class-specific proteinase inhibitors were tested to determine their ability to inhibit VV replication in tissue culture cells. To that end BSC40 tissue culture cells were infected with VV in the presence of various concentrations of proteinase inhibitors. Efforts were made to use concentrations of drugs which had minimal effects on the tissue culture cells as judged by morphological appearance and thymidine incorporation. Inhibitors tested included; 1,10-phenanthroline, a metalloproteinase inhibitor (and its non-chelating isomer, 1,7-phenathroline); iodoacetamide, a cysteine proteinase inhibitor; and pepstatin A, an aspartic proteinase inhibitor. Unfortunately, any and all serine proteinase inhibitors tested were acutely toxic to the host cell, perhaps not a surprising result given the ubiquity of this type of proteinase in mammalian cells. Interestingly, VV replication was completely blocked by 10 μM iodoacetamide or 1 μM 1,10-phenanthroline whereas 1,7-phenanthroline or pepstatin A had no effect. These results are consistent with a metalloproteinase and a cysteine proteinase both playing an essential role in the viral replicative cycle.

I7L

Although there are several lines of evidence that suggest that G1L protein may be a metalloproteinase, another candidate proteinase has been identified, the gene product of the I7L open reading frame. This protein was originally identified as a potential protease on the basis of homology to a ubiquitin-like proteinase in yeast [71]. I7L is believed to belong to the SUMO-1- specific family of proteases, which includes the proteases encoded by adenovirus and African swine fever virus (ASFV). Interestingly these last two proteases cleave polyproteins at Gly-Gly-Xaa sites, similar yet distinct from the Ala-Gly-Xaa sites cleaved by I7L. Like G1L, I7L is highly conserved amongst the Orthopoxviruses, with 95–99% identity of the gene between the viruses in this family. It is predicted to be a cysteine proteinase and two potential active sites are evident. Condit and coworkers have isolated a temperature sensitive mutant in the I7L gene [72]. At the non-permissive

temperature, the core protein precursors P4a, P4b and P25K are synthesized but are not processed. Moreover, viral assembly is halted between immature viral particle formation and conversion to an infectious IMV particle [73]. At the non-permissive temperature no infectious progeny are produced [74].

I7L is predicted to encode a 47 kDa protein that is expressed at late times postinfection. Use of monospecific anti-I7L antisera has demonstrated that the protein is associated with virus factories, immature viral particles and IMV, where it is exclusively located in the core [73].

In order to determine which enzyme is the vaccinia virus core protein proteinase (vCPP), a transient expression assay was used to demonstrate that the I7L gene product and its encoded cysteine proteinase activity is responsible for cleavage of P25K, the product of the L4R open reading frame. Cleavage was demonstrated to occur at the authentic Ala-Gly-Xaa cleavage site and require active enzyme, as mutation of either the cleavage site or the active site residues in I7L abolished this activity. [75]

Having established that I7L was the vCPP, there were several questions that remained to be answered. It was not known whether the entire I7L protein was required for recognition and cleavage of the core precursor proteins, or if just the predicted catalytic domain was required? Was I7L capable of cleaving each of the core protein precursors, and did cleavage occur preferentially at Ala-Gly-Ala versus Ala-Gly-Ser and Ala-Gly-Thr sites? Was there a catalytic triad and were other conserved residues essential for activity? The results obtained from *trans* processing assays showed that intact I7L is necessary and sufficient to direct cleavage of each of the three major core protein precursors and that mutagenesis of either the putative catalytic triad of I7L or of the Ala-Gly-Xaa sites in the precursor proteins abolishes this activity. A series of truncated I7L proteins lost the ability to cleave the core protein precursors. [76]

In order to further characterise I7L, a conditional-lethal recombinant virus was constructed in which the expression of the vaccinia virus I7L gene is under the control of the tetracycline operator/repressor system. In the absence of I7L expression, processing of the major VV core proteins is inhibited and electron microscopy revealed defects in virion morphogenesis subsequent to the formation of immature virion particles but prior to core condensation. Plasmid-borne I7L was capable of rescuing the growth of this virus and rescue was optimal when the I7L gene is expressed using the authentic I7L promoter. [77]. In addition to cleaving the major core protein precursors, I7L has recently been shown to be capable of cleaving membrane proteins as well. Ansarah-Sobrinho and Moss [78] constructed a recombinant virus with I7L regulated by the *E. coli lac* repressor to demonstrate that in the absence of an inducer the A17 membrane protein is not cleaved at the canonical AGX cleavage site and viral morphogenesis is blocked. Taken together, these data suggest that correct temporal expression of the VV I7L cysteine proteinase is required for core protein maturation, virion assembly and production of infectious progeny. [77,78]

Following the identification and characterisation of I7L as an essential proteinase involved in the maturation of VV, it was then of interest to determine whether small molecule inhibitors could be developed to inhibit I7L and halt viral replication, which could then be used to inhibit each virus in this family including the causative agent of smallpox. Using a homology-based bioinformatics approach, a structural model of the vaccinia virus (VV) I7L proteinase was developed. A unique chemical library of $\sim$450 000 compounds was computationally queried to identify potential active site inhibitors. The resulting biased subset of compounds was assayed for both toxicity and the ability to inhibit the growth of VV in tissue culture cells. A family of chemotypically-related compounds were found which exhibited selective activity against orthopoxviruses, inhibiting VV with IC50 values of 3–12 μM. These compounds exhibited no significant cytotoxicity in the four cell lines tested, and did not inhibit the growth of other organisms such as *Saccharomyces cerevisiae*, *Pseudomonas aeruginosa*, adenovirus, or encephalomyocarditis virus (EMC), indicating the selective nature of the compounds. Phenotypic analyses of virus-infected cells were conducted in the presence of active compounds to verify that the correct biochemical step (I7L mediated core protein processing) was being inhibited. An electron microscopic examination of compound-treated VV infected cells indicated a block in morphogenesis with the same phenotype as the temperature

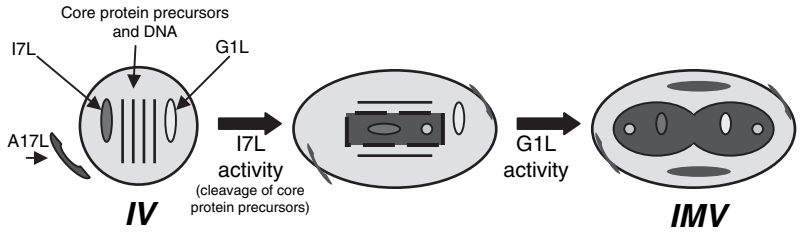

Figure 5. Model of the role of proteolysis in vaccinia virus morphogenesis. During the transition from an IV to an IMV particle there are a series of proteolytic cleavage events including the cleavage of the major core protein precursors by I7L and followed by the activity of G1L to lead to infectious virus particles.

sensitive I7L virus and the I7L conditional lethal virus in the absence of inducer, that is, the formation of immature viral particles but a lack of complete core condensation and no mature virus particles. To further demonstrate that the compound was inhibiting I7L and not another stage in the virus life cycle, compound resistant viruses were generated and resistance was mapped to the I7L ORF. Recombinant VV were then generated in which the wild type I7L ORF was replaced with the mutant I7L from the resistant virus. These recombinant viruses demonstrated resistance to the compound indicating that this was the only gene necessary for resistance and validated I7L as the drug target. This novel class of inhibitors demonstrated the potential for the development of VV proteinase inhibitors an efficient antiviral drug target. [79]

Finally, an *in-vitro* cleavage assay was developed to further characterize the activity of I7L in a cell-free system and show that the proteinase activity observed until now was the result of I7L specifically and not another viral or cellular protein. Using this assay, which is based on producing the major core protein precursors as substrates in a coupled transcription and translation assay and then mixing them with I7L enzyme extracts, I7L was shown to be capable of cleaving each substrate. A time course of activity at various temperatures was performed to determine the optimal temperature for *in-vitro* activity. Antibody pull-down studies showed that I7L specific antiserum could competitively inhibit the cleavage reaction, additionally verifying I7L as the enzyme responsible for activity. I7L was further characterised as a cysteine proteinase due to the inhibitory effects of known cysteine proteinase inhibitors such as NEM and iodoacetic acid, as well as through the use of specific small molecule inhibitors in this *in-vitro* assay. [80]

Taken together, the data presented here, as well as analysis of the VV G1L conditional lethal mutant [69], suggests a morphogenesis model in which these two putative proteases operate sequentially to regulate viral assembly, with I7L functioning to cleave the major core protein precursors to allow them to assume the proper configuration for virion maturation. This activity is followed by the action of G1L to allow for complete core condensation and the progression to the formation of intracellular mature virus particles (Figure 5). If the activity of the I7L proteinase is blocked, viral morphogenesis arrests prior to core condensation. If the activity of G1L is blocked, viral morphogenesis arrests at a stage subsequent to this but still prior to complete core condensation.

There are still several questions that remain unanswered about the activity of I7L including whether it has another role in the viral life cycle aside from cleavage of the major core protein precursors, what activates the enzyme, and how it is regulated so that it acts at a specific time in the viral life cycle. The role and identity of other vaccinia specific proteases has yet to be determined. However, the information provided here has shown that I7L is the first characterised vaccinia virus protease and an attractive and viable target for antiviral drug development. It will be of interest in the future to determine the specific activity of the G1L enzyme to complete the picture of vaccinia virus proteolysis.

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DDR

Research Overview

Viral Proteinases: Targets of Opportunity

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Strategy, Management and Health Policy				
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ABSTRACT During antiviral drug development, any essential stage of the viral life cycle can serve as a potential drug target. Since most viruses encode specific proteases whose cleavage activity is required for viral replication, and whose structure and activity are unique to the virus and not the host cell, these enzymes make excellent targets for drug development. Success using this approach has been demonstrated with the plethora of protease inhibitors approved for use against HIV. This discussion is designed to review the field of antiviral drug development, focusing on the search for protease inhibitors, while highlighting some of the challenges encountered along the way. Protease inhibitor drug discovery efforts highlighting progress made with HIV, HCV, HRV, and vaccinia virus as a model system are included. Drug Dev. Res. 67:501–510, 2006. © 2006 Wiley-Liss, Inc.

Key words: proteinase inhibitors; antiviral drugs; vaccinia virus; I7L

INTRODUCTION

Traditional antiviral strategies have relied on the use of vaccines to prevent viral infection or the use of rest and supportive therapy when a person did become infected since specific antiviral agents were not available. However, there are many virus infections for which a vaccine is not available or for which vaccine development may be problematic due to the virus having many serotypes, so the discovery of antiviral drugs has become a high priority. Initially, the discovery of antiviral drugs was based on screening large chemical libraries for compounds that would inhibit viral replication, and this effort met with relatively little success. The development of new antiviral agents is now making rapid progress due in large part to the recent advances in research on specific virus families. These advances include the advent of sequencing of entire viral genomes, discovery of permissive cell lines, replicon systems, and pseudotype viruses, as well as improvements in rational drug design and combinatorial chemistry. Until the 1980 s, amantadine was the only approved antiviral drug, authorized by the FDA for the treatment of influenza A in 1966 (http://www.fda.gov/cder/drug). However, the past 20 years have seen the development of new antiviral agents targeting various stages of the viral life cycle for a number of viruses including human immunodeficiency virus (HIV), hepatitis B and C viruses, herpes simplex virus (HSV), cytomegalovirus (CMV), varicella-zoster virus (VZV), influenza A and B viruses, and respiratory syncytial virus (RSV) (Table 1). Additional antiviral drug examples include the use of interferon for human papilloma virus (HPV) [Cantell, 1995].

Antivirals are most commonly used against active viral disease. However, prophylactic use is becoming

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TABLE 1. Antiviral Agents Approved for Use in the United States

Stage of virus life cycle	Antiviral agent	Approved	Target viruses
Entry and uncoating	Enfuvirtide	2003	HIV
	Amantadine	1966	Influenza A
	Rimantadine	1993	Influenza A
Replication	Azidothymidine (AZT)	1987	HIV
	Didanosine	1991	HIV
	Zalcitabine	1992	HIV
	Stavudine	1994	HIV
	Lamivudine	1995	HIV. HBV
	Nevirapine	1996	HIV
	Delavirdine	1997	HIV
	Abacavir	1998	HIV
	Efavirenz	1998	HIV
	Tenofovir	2001	HIV
	Adefovirn dipivoxil	2002	HBV
	Emtricitabine	2003	HIV
	Acyclovir	1997	HSV, VZV
	Foscarnet	2005	CMV, HSV, VZV
	Ganciclovir	2003	HSV, CMV
	Penciclovir	1996	HSV
	Trifluridine	1995	HSV
	Valacyclovir	1995	HSV, VZV
	Cidofovir	1996	CMV
	Ribavirin	1980	RSV, HCV, Lassa
Protease inhibitors	Saquinavir	1995	HIV
	Ritonavir	1996	HIV
	Indinavir	1996	HIV
	Nelfinavir	1997	HIV
	Amprenavir	1999	HIV
	Lopinavir	2000	HIV
	Atazanavir	2003	HIV
	Tipranavir	2005	HIV
Release	Oseltamivir	1999	Influenza A, B
	Zanamivir	1999	Influenza A, B

increasingly recognized as a viable strategy especially for those at risk of contracting infection such as immunocompromised individuals, organ transplant recipients, elderly patients, those in a "hot zone" near an outbreak, military personnel, and travelers to areas where the virus may be endemic.

One of the main challenges to the development of a successful antiviral drug is specificity, to identify a target that is specific to the virus and not to the host cell. Common stages of the virus life cycle to target are attachment and entry, replication, assembly, and release. Regardless of the stage of the life cycle targeted, the drug must be more toxic to the virus than to the host cell. To date, the majority of the approved antiviral agents are nucleoside analogs that inhibit viral DNA synthesis. While many of these drugs are highly effective, their continued use leads to the emergence of drug-resistant viral strains, so the development of new antiviral agents with a different virus-specific target is necessary. Entry inhibitors are a promising class of antivirals with several drugs approved for use in humans, such as enfuvirtide for HIV [Burton, 2003; Robertson, 2003] and amantadine for influenza A [Nahata, 1987]. However, susceptibility and the development of resistance may still be a significant hurdle to overcome. For example, in the case of HIV, Env is the most variable HIV protein [Weiss et al., 1986; Yasunaga et al., 1986], and the susceptibility of each virus strain to specific entry inhibitors may vary. It is possible that the development of mutations leading to resistance to some entry inhibitors may affect viral tropism and pathogenicity. Viral enzymes that are essential for the production of infectious progeny virus are an attractive target and recent advances in molecular biology, structural biology, computational biology, and biochemistry are making protease inhibitors an attractive viral target. While there is usually some variability in the coding region of proteases, the active-site region is usually very highly conserved, making it likely that a drug that targets the active site of a protease will inhibit all serotypes, while a drug targeting a less highly conserved viral protein would have more variability. This has been demonstrated with the success of rupintrivir, a novel inhibitor of the 3C protease of human rhinovirus (HRV), in being effective against each of the picornaviruses that were tested, including almost 100 HRV and human enterovirus (HEV) serotypes [Binford et al., 2005].

PROTEASES

Studies during the last 20 years have shown that viral proteases, enzymes that selectively cleave polypeptide bonds, are absolutely essential during the life cycle of many viruses [Dougherty and Semler, 1993; Kay and Dunn, 1990; Krausslich and Wimmer, 1988]. Proteases can be either peptidases, which cleave single amino acids from the end of a peptide chain, or proteinases, which cleave peptide bonds within a substrate [Polgar, 1989]. Viral proteinases function either to cleave high-molecular-weight viral polypeptides into functional protein products during formative proteolysis or to cleave structural proteins necessary for assembly during morphogenic proteolysis. Proteinases can be further divided into four separate categories (serine, cysteine, aspartic, and metalloproteinases) based on the identity of their catalytic residues, the mechanism of catalysis, and their substrate specificity. For a general review of each type of proteinase, see Dougherty and Semler [1993] and Barrett et al. [2004]. Serine proteinases have a catalytic triad composed of

serine, histidine, and aspartic acid and usually have an accompanying oxyanion hole intermediate. The active serine hydroxyl carries out a nucleophilic attack of the carbonyl group of the amide bond. Serine proteases can be categorized based on substrate specificity as being trypsin-like, chymotrypsin-like, or elastase-like. Cysteine proteinases are similar in amide bond hydrolysis to serine proteases, but have a catalytic triad composed of cysteine, histidine, and either asparagine or aspartic acid. Cysteine proteinases have been categorized as papain-like, interleukin-1 beta-converting enzyme (ICE)-like, or picornaviral (which are similar to serine proteases but with a cysteine instead of serine). Aspartic proteinases contain an active site composed of two aspartic acid residues and generally bind 6–10 amino acids of their substrate [Leung et al., 2000]. Catalysis is through an acid-base mechanism where a deprotonated catalytic aspartic acid residue activates a water molecule, which then carries out a nucleophilic attack on the scissile bond. Finally, metalloproteinases use a zinc atom to effect amid bond hydrolysis. Serine, cysteine, and aspartic proteinases have been well characterized in viral systems [Hellen and Wimmer, 1992; Kay and Dunn, 1990]. However, although examples of viral proteinases that coordinate a zinc atom during catalysis have been described [Love et al., 1996], there are currently no known viral examples of true metalloproteinases. One possible exception to this may be the G1L protein of vaccinia virus, which has been predicted, although not yet proven, to be a metalloproteinase [Ansarah-Sobrinho and Moss, 2004b; Byrd et al., 2004b; Hedengren-Olcott et al., 2004; Whitehead and Hruby, 1994].

Protease inhibitors work by binding either to the active site of the enzyme or to the substrate-binding groove to inhibit the ability of the enzyme to either recognize its substrate or to cleave it. Inhibition can be either direct (by directly competing with the substrate) or indirect (by competing with a non-catalytic cofactor).

History of protease inhibitor discovery

Drug discovery and development efforts have changed a great deal over the past few decades to become more target specific and less toxic. Originally, drug discovery was largely centered on screening available compound libraries or natural products for compounds that would inhibit viral replication in tissue culture, without the exact mode of viral inhibition being known. As more information became available about the roles of specific essential enzymes in the viral life cycle, there was a shift in focus to look for specific inhibitors. With protease inhibitors, drug design began with looking at substrate-derived products and peptides normally cleaved by the protease. Peptides can serve as inhibitors in several ways, one of which is by replacing the scissile bond in the natural substrate peptide with a non-cleavable bond as has been demonstrated for HCV [Ingallinella et al., 2000], or through product inhibition since many of the products of protease-catalyzed reactions naturally serve as inhibitors when present in the reaction mixture such as that observed with the HCV NS3 protease [Steinkuhler et al., 1998]. By introducing structural modifications to shorten the peptide, make it non-cleavable, and increase potency, these peptides could be optimized as inhibitors. While natural peptide substrates have the benefit of providing information about molecular interactions with proteases and can, therefore, provide clues to inhibitor design, there are some significant drawbacks to their use as antivirals. Unfortunately, the use of peptides as a drug is often limited by issues of instability and poor pharmacokinetic profiles. Peptides can be susceptible to degradation and fast metabolism, low membrane permeability, low oral bioavailability, and quick elimination from plasma [Ghosn et al., 2004; Hostetler et al., 1994; Kempf et al., 1991; Matsumoto et al., 2001]. Peptide drugs also have the potential to induce an immunogenic response, which may lead to a loss of drug efficacy or adverse events in the recipient. However, PEGylation, the addition of one or more polyethylene glycol (PEG) chains, can reduce immunogenicity and degradation of the peptide drug [Veronese and Pasut, 2005]. In addition to specificity, bioavailability, and membrane permeability, for a drug to be successful it should have acceptable toxicity, absorption, distribution, metabolism, and excretion profiles.

Fortunately, with the availability of the three-dimensional structure of many proteinases determined by X-ray crystallography and NMR, drug discovery has progressed into mechanism-based drug design. By using computer-assisted structure-based design, small molecule compounds could be queried for their ability to fit into the active site pocket of the proteinase in silico. Small molecule inhibitors have the advantage of being more orally bioavailable, permeable, selective, often work in the micromolar or sub-micromolar range, have few or no hydrolysable bonds, can be easily modified through combinatorial chemistry, and can have reduced production costs over peptide inhibitors. This rational drug design approach has the benefit of looking at many parameters of the molecule/protease interaction to improve the inhibitory activity and improve the pharmacokinetic (PK) profile of the compound. Small molecule compounds identified through in silico screening are then screened in vitro to look for their ability to inhibit the proteinase target and viral replication in general. Combinatorial chem-

istry can then be used to optimize the compound into a lead drug.

SPECIFIC EXAMPLES

HIV provides an excellent example of the success of antiviral drug treatment as the virus was isolated in 1983 and the first treatment (AZT) was approved for use only 4 years later in 1987. The first HIV antivirals approved for use were nucleoside analogs, which target reverse transcriptase (RT) to inhibit viral replication. However, their monotherapeutic use had only moderate clinical efficacy, was limited by adverse side effects, and rapidly generated highly resistant drug variants making the search for new inhibitors necessary. Enfuvirtide, a novel 36-amino-acid synthetic peptide entry inhibitor, has shown promise in clinical trials and has been approved for use, but remains very expensive to manufacture and has to be administered by frequent injections [Chen et al., 2002; Hanna, 1999; Lalezari et al., 2003; Robertson, 2003; Steinbrook, 2003]. The HIV protease, an aspartic protease, has become an extremely attractive target for antiviral drug development with great success. The crystal structure has been solved and several of the protease-inhibitor crystal structures are available at www.rcsb.org/pdb. Several low molecular weight inhibitors have been approved for use in humans and are among the first successful examples of structure-based drug design that target the active site substrate-binding groove of the enzyme (Table 1). Beginning with saquinavir, which was approved in 1995, protease inhibitors for HIV have been developed with improved oral bioavailability, plasma concentrations, and increased half-life, which allow for less frequent administration of the drug and therefore less adverse side effects. Saquinavir suffered from low oral bioavailability, but was quickly supplemented with ritonavir in 1996, which had a high oral bioavailability [Vella, 1995]. The success of these protease inhibitors was followed by the development of indinavir, nelfinavir, aprenavir, lopinavir, atazanavir, and tipranivir. Indinavir required precise dosing every 8 h. However, as improvements were made in protease inhibitors, dosing requirements decreased, with aprenavir allowing twice a day dosing, and atazanavir being the first HIV protease inhibitor to allow once a day dosing. Ritonavir is unique amongst the approved HIV protease inhibitors in that it inhibits the host liver enzyme cytochrome P450-3A4 (CYP3A4) [Kumar et al., 1996], which normally metabolizes other protease inhibitors. Ritonavir is, therefore, mainly used in combination with other protease inhibitors since it decreases their metabolism. The rest of the HIV protease inhibitors target the active site substrate-binding groove of the enzyme. Nelfinavir and tipranavir are the only two non-peptidic HIV protease inhibitors. Table 2 highlights some of the major drug-resistant mutations in the protease gene of HIV-1 [Johnson et al., 2005] developed in response to the various protease inhibitors, pointing out where cross-resistance between drugs is likely. Not indicated in Table 2 are many of the minor mutations that appear in drug-resistant isolates that by themselves do not cause drug resistance but may improve fitness of viruses containing another major mutation. Newly emerging drug-resistant strains of HIV intensify the need for more antiviral drugs. Recently, a new morphogenesis inhibitor, PA 457, which appears to act on the substrate of the proteinase instead of the enzyme active site, is in phase II clinical trials and has been granted fast-track status by the FDA [Reeves and Piefer, 2005].

Most aspartic protease inhibitors developed to date have been found through either screening compound libraries or through rational drug design and bind to the protease through non-covalent interactions, making them reversible inhibitors that must show greater affinity for the protease than the protease for its natural substrate. This high affinity is achieved through rational drug design by increasing the number of interactions between the inhibitor and enzyme. There are currently 8 different HIV protease inhibitors

TABLE 2. Major Drug-Resistant Mutations in the Protease Gene of HIV-I

	D30N	V32I	L33F	M46I/L	I47V/A	G48V	150L/V	V82A/F/T/ V/S/L	184V	N88S	L90M
Saquinavir						X					X
Ritonavir								X	X		
Indinavir				X				X	X		
Nelfinavir	X										X
Amprenavir							X		X		
Lopinavir/ritonavir		X			X			X			
Atazanavir							X		X	X	
Tipranavir/ritonavir			X					X	X		

on the market that can be used in combination therapy to increase antiviral potency and decrease the chances of the development of resistance.

Although the only currently approved protease inhibitors are for HIV, there are several very promising protease inhibitors currently in clinical trials for a variety of other viruses including inhibitors of the NS3 serine protease of HCV. First identified in 1989, HCV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Since the virus establishes a chronic infection, and long-term treatment may be necessary, a combination drug approach such as that used for HIV may be required to avoid problems of resistance. Until recently, drug discovery efforts for HCV were hindered by factors such as the persistence of the virus in the host, which can lead to genetic diversity, development of drug resistance, and lack of good cellular and in vivo models with which to screen antiviral compounds [Magden et al., 2005]. Initial efforts to overcome these problems were benefited by the development of a replicon system that relies on stable replication of subgenomic RNAs in human hepatoma cells [Lohmann et al., 1999] and the creation of pseudotype viruses expressing HCV glycoproteins [Bartosch et al., 2003; Hsu et al., 2003; Lagging et al., 1998]. While useful for studying and targeting specific viral proteins, these systems still lacked the production of infectious virus. The recent identification of cell lines that produce infectious HCV virus [Cai et al., 2005; Kanda et al., 2006; Valli et al., 2006; Yi et al., 2006] will allow significant improvements in the ability of researchers to study the virus life cycle and look for antiviral drugs. BILN 2061, an orally bioavailable small molecule inhibitor, showed initial promise in human trials with a reduction of HCV RNA in the plasma, and established the proof-of-concept in humans that a HCV protease inhibitor can be effective [Lamarre et al., 2003]. Unfortunately, due to studies that showed that BILN 2061 may cause heart damage in animal models, further progress with this particular drug was halted [Hinrichsen et al., 2004]. However, the FDA has granted Fast Track development status to two other HCV protease inhibitors, Schering-Plough's oral protease inhibitor SCH 503034 and Vertex's VX-950, which are both in Phase II clinical trials. The current treatment for HCV is a combination of ribavirin with PEG-interferon alpha and, while efficient, it does cause significant toxicity, and many patients do not respond to treatment, prompting the search for other antivirals.

Significant progress has also been made in the search for protease inhibitors against the human rhinovirus (HRV) 3C cysteine protease. The HRV protease is a 20-kDa picornavirus protease with homology to trypsin-like serine proteases. The HRV 3C protease selectively catalyzes the peptide bond after the Gln residue in the Val/Thr-X-X-Gln-Gly-Pro consensus sequence [Long et al., 1989]. With the determination of the 3D structure of the HRV 3C proteinase [Matthews et al., 1994], progress in rational drug design against this target has been made. Structure-assisted design resulted in the discovery of rupintrivir, formerly AG7088, which has shown good selectivity and activity against all the serotypes tested [Binford et al., 2005]. Rupintrivir binds irreversibly to the active site cysteine and has shown promise in phase II clinical trials [Hayden et al., 2003]. Another orally bioavailable small molecule inhibitor of the HRV protease is being developed by Pfizer and has shown efficacy in cell-based assays and safety in human trials [Patick et al., 2005]. Like HIV, the specificity of protease inhibitors may prove to be critical for HRV inhibitors. The small molecule inhibitor pleconaril, which inhibits the viral uncoating process, has recently shown efficacy in reducing the symptoms associated with upper respiratory tract disease [Pevear et al., 2005]. However, in two clinical trials 13% of isolates were not susceptible to the drug, demonstrating a limit in the spectrum of activity of some entry inhibitors [Fleischer and Laessig, 2003]. The FDA did not grant permission to use pleconaril to treat the common cold due to some evidence that it interfered with the action of contraceptives (www.fda.gov/ohrms/dockets/ac/02/briefing/ 3847b1_02_FDA.pdf).

CHALLENGES TO DEVELOPMENT

The development of a successful antiviral drug has many challenges to overcome before being approved for use in humans. The first and often the most difficult challenge can be assay development, designing a suitable system to study the effects of an antiviral both in vitro and in vivo. Some viruses have not been adapted to grow in tissue culture cells or due to their genetic makeup are difficult to engineer or manipulate. Creative solutions to these issues are exemplified by research around HCV, which led to the use of replicon systems and pseudotype viruses to be able to study specific aspects of the virus life cycle.

Once a suitable assay is developed to screen specific inhibitors, the challenge can then be with rational drug design. If the crystal structure of a protease is not available, homology modeling must be relied on to approximate the three-dimensional structure of the enzyme. However, this method can still be reasonably reliable, especially if there is significant similarity in the active site region of the protein being targeted and a homolog with a known 3D-structure. As molecules are being screened for their ability to interact with this active site pocket (or a secondary

allosteric binding site), specificity again becomes an issue. Reversible inhibitors, which make hydrogen bonds, ionic, and van der Waal's interactions, are generally preferred over irreversible inhibitors, which usually form a covalent bond between enzyme and inhibitor since irreversible inhibitors could be expected to covalently bind with many proteins before encountering the target protein and thus result in more toxic side effects.

After compound inhibitors are screened in a suitable in vitro assay, whether biochemical or cell based, the compound should then be tested against a variety of viruses for specificity and against a number of cell lines for toxicity. Those compounds that show good activity and specificity with a lack of toxicity need to be tested in an appropriate animal model to determine both the pharmacokinetic profile of the drug as well as its effectiveness in preventing or treating disease. Determining an appropriate animal that approximates the human disease can prove quite challenging, especially for viruses that normally only infect humans such as HIV and those that are rare or naturally nonexistent such as variola, which still pose a bioterror threat. Even if an animal model is found that is predictive of the disease in healthy adults, it may not approximate the disease in immunocompromised individuals, infants, the elderly, or pregnant women.

In addition to the challenges surrounding drug development, there is a need for more rapid and specific diagnostics in order to use antiviral drugs. By the time most acute viral infections become evident, the symptoms are generally side-effects of the immune response and viral titers are already dropping, thus a drug would only be useful if you knew you were previously exposed. Also, many different viruses can cause the same disease. For example, the common cold can be caused by rhinoviruses, adenoviruses, coronaviruses, and so on. Each virus has a different genome and different drug targets, so knowing exactly which virus is causing the infection is necessary to prescribe a specific antiviral drug.

VACCINIA VIRUS AS A MODEL SYSTEM

Ongoing studies with vaccinia virus (VV) demonstrate the process of antiviral drug discovery efforts. Though smallpox was eradicated in 1980, there has been concern in recent years that variola virus (the causative agent of smallpox), a related orthopoxvirus such as monkeypox virus, or a genetically engineered poxvirus may pose a threat as a bioterrorism agent. With the discontinuation of vaccination against smallpox, the subsequent 30 years have produced a population that is immunologically naive and susceptible to infection. With the potential adverse events associated with widespread use of the currently available vaccine, which can include myocarditis, pericarditis, generalized vaccinia, eczema vaccinatum, encephalitis, and neurologic illness [Casey et al., 2005; Cohen et al., 2006; Fulginiti et al., 2003; Sejvar et al., 2005], antiviral drugs against orthopoxviruses are actively being sought. An antiviral would be useful both with co-administration with the vaccine to limit adverse effects as well as a prophylactic or therapeutic for exposure to poxviruses. Since smallpox is a biosafety level 4 agent with the only available stocks in the United States at CDC, much of the antiviral research is done using a highly related virus, vaccinia.

VV is a large DNA virus with a cytoplasmic site of replication. The life cycle begins with attachment and entry of the virus through an as yet unknown mechanism, followed by uncoating to release the viral core that contains the viral genome and the viral transcriptional apparatus. Gene expression occurs in an orderly cascade with the products of early gene expression necessary for DNA replication, intermediate gene expression, and late gene expression. After the late genes are expressed, which encode many of the structural proteins and enzymes, assembly of the virus occurs in areas of the cytoplasm that are termed virosomes or virus factories. During assembly of immature viral particles, viral-encoded proteinases cleave the major core protein precursors during morphogenic proteolysis to lead to the first of four infectious forms of virus, intracellular mature virus (IMV). A portion of the IMV particles acquire an additional membrane(s) from the trans Golgi network to become intracellular enveloped virus (IEV). Whether IMV acquire one or two membranes during this transition is a well-debated issue. Regardless, the IEV then travel to the cell surface via actin tails to become either cell-associated enveloped virus (CEV) or are released to form extracellular enveloped virus (EEV). Figure 1 depicts the events that occur during the VV life cycle along with potential targets for antiviral drug development. Initially, the search for an effective orthopoxvirus antiviral focused on looking at compounds that are already approved for use against other indications. While these efforts have identified compounds that have proven useful for studying the replication of the virus in vitro, they have not provided antivirals that are effective in vivo either due to toxicity or lack of effectiveness against pox viruses. Stages of the life cycle that have been targeted include DNA synthesis, transcription, morphogenesis, and exit of IMV and EEV from the cell. Examples of some of these are listed in Table 3. The DNA synthesis inhibitor cytosine arabinoside (AraC), which is used to treat some leukemia, inhibits VV replication [Herrmann,

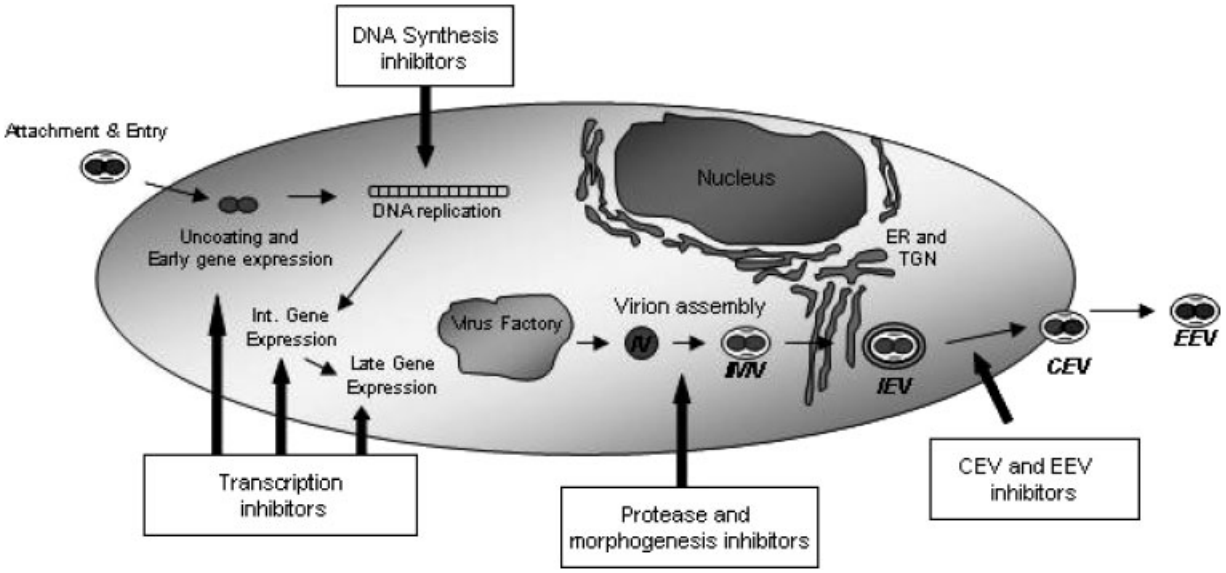

Fig. 1. Vaccinia virus life cycle. Stages of the life cycle essential for replication that make good targets for drug development are indicated and include DNA synthesis, transcription, morphogenesis, and exit from the cell.

TABLE 3. Compounds That Inhibit Vaccinia Virus Replication In Vitro[a]

Stage of VV life cycle targeted	Drug	Used for	Adverse effects
DNA synthesis	AraC	Leukemia	Bone marrow suppression, cerebellar toxicity
	Hydroxyurea	Hematological malignancies	Bone marrow toxicity
	Cidofovir	CMV retinitis in AIDS	Lack of oral bioavailability
	Ribavirin	RSV, HCV, Lassa	Not effective against pox in vivo
Transcription	Distamycin	Antibiotic	Thrombophlebitis, bone marrow toxicity
Morphogenesis	Rifampicin	Mycobacterium	Hepatotoxicity
	Novobiocin	Staphylococcus epidermidis	Hepatotoxicity and blood dyscrasias
	IMCBH	Pox viruses	Not effective in animal models
	TTP-6171	Novel compound	Not effective in animal models
CEV and EEV	ST-246	Novel compound	No adverse effects→in clinical trials

[a]Stage of VV life cycle targeted, what the compound is commonly used for, and some adverse effects that may limit its use are indicated. AraC, cytosine arabinoside; IMCBH, N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine.

1968] but causes bone marrow suppression, leucopenia, and cerebellar toxicity in humans [Barista, 2000] so its use is mainly limited to cancer chemotherapy. Hydroxyurea, which is used for hematological malignancies [Johnson et al., 1992] also acts as an antiviral by inhibiting ribonucleotide reductase and inhibits vaccinia virus at an early stage [Rosenkranz et al., 1966]. However, it can cause bone marrow toxicity, hair loss, and skin changes [De Benedittis et al., 2004].

The other approach to orthopoxvirus antiviral drug discovery is to screen new chemical libraries for specific inhibitors of various stages of the virus life cycle. Recently, the small molecule compound ST-246 has shown to be effective in inhibiting EEV formation and is protective in several animal models [Yang et al., 2005]. ST-246 was discovered through a high-throughput screening assay looking at compounds that inhibited virus-induced cytopathic effects. Drug-resistant virus variants were created and used to map the location of resistance to the F13L gene in vaccinia virus, which encodes a protein necessary for the production of extracellular virus. This compound is currently in human clinical trails and shows promise for development as an antiviral drug.

Another very promising target is the recently discovered VV I7L proteinase, which is the viral enzyme responsible for cleavage of the major core protein precursors as well as membrane proteins and is absolutely essential for viral replication [Ansarah-Sobrinho and Moss, 2004a; Byrd and Hruby, 2005a].

While other proteinases, either cellular or viral, may be involved in the virus life cycle, to date I7L is the only characterized proteinase involved in the virus life cycle. I7L is a 47-kDa cysteine proteinase that cleaves its substrate proteins at a conserved Ala-Gly-Xaa motif [Byrd et al., 2002]. Mutagenesis of conserved residues within I7L and around the catalytic triad (H241, D258, C328) have helped define functional regions of the enzyme [Byrd et al., 2003]. Although efforts to date to express and purify active enzyme in vitro have not been successful, an in vitro assay to screen for potential I7L inhibitors has been developed [Byrd and Hruby, 2005b]. Homology modeling of the I7L proteinase, based on similarity to the C-terminal domain of the ULP1 protease in yeast, has been successfully used for in silico screening of a large chemical library of small molecules for potential inhibitors [Byrd et al., 2004a]. One of these, TTP-6171, showed initial promise against I7L and was a potent inhibitor of viral replication in vitro [Byrd et al., 2004a]. However further studies in animals showed less efficacy, highlighting the need for additional pharmacokinetic analysis and combinatorial chemistry (data not shown). I7L shares regions of similarity with several other viral proteinases including the African Swine Fever virus protease and the Adenovirus protease. However, there are no cellular homologs of I7L, making it likely that a drug that inhibits it will be highly specific for the virus and not the host cell. I7L remains an attractive target and efforts are ongoing in the search for an effective antiviral drug against this enzyme.

Some of the difficulties with antiviral drug research are exemplified through research with vaccinia virus. Once an attractive target is discovered, such as the I7L core protein proteinase, biochemical and whole cell assays need to be set up and validated to screen inhibitory compounds. Compounds can be identified as potential inhibitors either through rational drug design, using homology modeling of the enzyme, since the crystal structure has not yet been solved, in combination with in silico screening of compound libraries, or through high-throughput screening of large chemical libraries. Once a compound has been identified as an inhibitor of the I7L enzyme through biochemical assays, its ability to inhibit viral replication needs to be validated in tissue culture both against vaccinia virus as well as other orthopoxviruses. Of particular interest to the development of poxvirus antivirals is the need to satisfy the animal efficacy rule set up by the FDA to evaluate new drug candidates. This can be a unique problem with smallpox antivirals since the disease is no longer endemic, and animal models of disease require the use of surrogate viruses, which do not closely mimic the human disease. A review addressing these concerns as well as how to select appropriate animal models has recently been published [Jordan and Hruby, 2006].

CONCLUSIONS

Modern molecular biology has provided the tools necessary to study the details of viral replication and has allowed the identification and development of many specific antiviral drugs. However, the problems of toxicity and resistance that occur with long-term use of many of these antiviral agents remain, not to mention that many of the approved antivirals have a narrow spectrum of activity and limited therapeutic usefulness. Combine this with the ongoing identification and characterization of newly emerging virus infections, and it can be seen that there are a very limited number of effective antiviral compounds against a small number of viruses. While significant progress in antiviral drug development has been made in the last several years, there is a clear gap in our arsenal of available antiviral agents, and the continued search for new compounds and strategies is essential.

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Short report

Analysis of vaccinia virus temperature-sensitive I7L mutants reveals two potential functional domains

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Abstract

As an approach to initiating a structure-function analysis of the vaccinia virus I7L core protein proteinase, a collection of conditional-lethal mutants in which the mutation had been mapped to the I7L locus were subjected to genomic sequencing and phenotypic analyses. Mutations in six vaccinia virus I7L temperature sensitive mutants fall into two groups: changes at three positions at the N-terminal end between amino acids 29 and 37 and two different substitutions at amino acid 344, near the catalytic domain. Regardless of the position of the mutation, mutants at the non-permissive temperature failed to cleave core protein precursors and had their development arrested prior to core condensation. Thus it appears that the two clusters of mutations may affect two different functional domains required for proteinase activity.

Findings

Vaccinia virus (VV) is the prototypic member of the orthopoxviruses, a genus of large, double-stranded DNA viruses which includes the human pathogens variola virus and monkeypox virus. VV has a complex replication cycle where, as in many other viruses, proteolysis plays a key role in the maturation process. The initial step in virion assembly is envelopment of viroplasm by crescent shaped membranes to form immature virions (IV). The IVs must then undergo a series of morphological changes, including cleavage of a number of core protein precursors, to become intracellular mature virions (IMV), the first of several different infectious forms.

The product of the VV I7L open reading frame (ORF) has been shown to be the viral core protein proteinase responsible for cleavage of the major core protein precursors P4a (A10L), P4b (A3L), and P25K(L4R) [1,2]. It is a cysteine proteinase, with a catalytic triad consisting of a histidine, an aspartate and a cysteine residue [2] and cleaves its substrates at conserved AG*X motifs [3-5]. In addition to the major core protein precursors, I7L has been shown to cleave the membrane protein A17L [6] and may also be responsible for the cleavage of other viral proteins containing the AG*X motif such as A12L and G7L whose cleavage has been documented but not attributed to a particular proteinase [5,7].

In the absence of functional I7L, virion morphogenesis is irreversibly arrested after the formation of IV but prior to the formation of IMV [6,8,9]. Despite the potential importance of this enzyme, relatively little is known about the biochemistry of the cleavage reaction or the structural features which allow I7L to direct regulated catalysis. Up to this point, all attempts to produce purified, functional I7L have failed, thereby limiting progress in this area. An alternative approach for studying the I7L protein is an analysis of the existing collections of temperature-sensitive (ts)

mutants. Six ts mutants from the Dales and Condit collections have been identified as I7L mutants using complementation analysis [[10] and S. Kato, T. Bainbridge, N. Moussatche, and R. Condit, personal communications]. Using the classification system proposed by Lackner et al. (with the original Dales designations in parenthesis), these are: Cts-16, Cts-34. Dts-4 (260), Dts-8 (991), Dts-35 (5804), and Dts-93 (9281). Though both collections were created by chemical mutagenesis, the Condit mutants were derived from the commonly used strain Western Reserve (WR) [11,12], while the Dales mutants were derived from the strain IHD-W, an IHD-J subtype [13].

Of the six mutants, Cts-16 has been the best studied and most frequently used, primarily as a means to establish a viral infection in the absence of functional I7L. Originally it was classified as having a wild type pattern of protein synthesis [11], although it was later shown that while the major core protein precursors are synthesized, they are not cleaved at the non-permissive temperature [14]. In Cts-16, I7L has also been shown to be stably produced at the non-permissive temperature [9] and is probably included in the core. The core protein precursors also localize normally at the non-permissive temperature [14].

Dales grouped his mutants into categories based on the apparent level of development attained as determined by electron microscopy. He classified Dts-8 as a category L mutant ("immature particles with nucleoids and defective membranes with spicules") and Dts-35 as category O ("immature normal particles and mature particles with aberrant cores") [13]. Using his classification system, Cts-16 best fits category K ("granular foci and immature particles with nucleoids but lacking internal dense material") or category L. Dales did not assign Dts-93 to a category while Dts-4 was not included in the original publication. Cts-34 has also not been described other than as an I7L mutant.

In order to further characterize these ts viruses and to determine the exact location of the mutation or mutations within the I7L ORF of each virus, genomic DNA was extracted from each virus type. The I7L ORF was PCR-amplified using the primers CB26 and CB90 [15], and the same primers used to sequence the purified PCR products. Multiple copies of the sequence of the WR I7L ORF have been deposited with GenBank [GenBank: AY49736, GenBank:AY243312, and GenBank:J03399] and were obtained for this analysis.

Sequencing of the parental strain IHD-W revealed two differences as compared to the I7L ORF of WR with arginine instead of lysine at amino acid (aa) 287 and glutamine instead of histidine at aa376 (Figure 1). WR is reported to have either aspartate or asparagine at aa420 while IHD-W has asparagine. The I7L ORF sequence from IHD-W was identical to that of Dts-97, a mutant in the E9 ORF [S. Kato, T. Bainbridge, N. Moussatche, and R. Condit, personal communications]. When these polymorphisms are taken into account, all the I7L ts mutants contain a single amino acid change. Cts-16, as previously reported and reconfirmed in our stock, has a proline to leucine change at aa344 [9]. Cts-34 has glycine to glutamate at aa29, Dts-4 has serine to phenylalanine at aa37, Dts-8 has proline to serine at aa344, and both Dts-35 and Dts-93 have aspartate to asparagine at aa35. Interestingly, the mutations seem to form two clusters with Cts-34, Dts-4, Dts-35, and Dts-93 containing three different mutations in a stretch of nine amino acids at the N-terminal end and Cts-16 and Dts-8 representing two different mutations in a single amino acid located toward the C-terminus and just downstream of the catalytic cysteine. The possible significance of these groupings is discussed below.

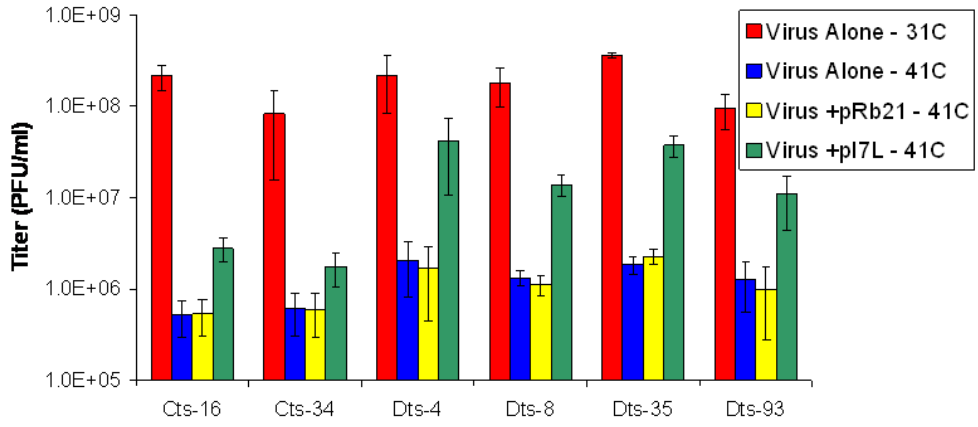
Since the mutants were created by chemical mutagenesis, there is the possibility of second-site mutations that contribute to the observed phenotype. To check for this, we attempted to rescue the replication of each virus with plas-

Figure 1
Schematic diagram of the I7L open reading frame. The amino acid changes found in the temperature sensitive mutants are represented above while the parental polymorphisms are given below. Black bars represent the putative catalytic triad.

mid born I7L. Using DMRIE-C (Invitrogen), BSC_{40} cells were transfected with 2 μg of either the empty vector pRB21 [16] or pI7L [8], infected at a multiplicity of infection (MOI) of 2, and incubated at the non-permissive temperature of 41C. pI7L contains the I7L ORF under the control of its native promoter and has been shown to give more efficient rescue than I7L under the control of a synthetic early/late promoter [8]. Mock transfected cells were also infected at an MOI of 2 and incubated at either 41C or the permissive temperature of 31C. The cells were harvest at 24 hours post infection (hpi), resuspended in 100 μl PBS and subjected to three freeze-thaw cycles. These lysates were titered onto confluent BSC_{40} cells in a series of 10-fold dilutions. After 48 hours of incubation at 31C, plaques were visualized by staining with 0.1% crystal violet.

All the ts mutants were rescued by the plasmid containing I7L, while transfection with an empty plasmid caused no increase in viral titer (Figure 2). This indicates that for each virus the mutation within the I7L ORF is the primary, if not only, cause of their temperature sensitive phenotype. Transfection with pI7L resulted in a 2.9 to 20.7 fold increase in viral titer over virus alone at the non-permissive temperature, causing the viruses to reach between 1.3 and 19.1% of their permissive temperature titer. Cts-34 showed the weakest rescue with a fold increase in titer only about half that of the next lowest value. However, as discussed below, its electron microscopic appearance and cleavage activity were identical to those of the other mutants, indicating that even if a second-site mutation exists, the affected protein acts with or after I7L. The degree of leakiness was low for all the mutants with the non-permissive temperature titer being 1.4% or less than that of the permissive temperature titer. As such, leakiness is not expected to have significantly affected the experiments.

Since I7L has been implicated as the core protein proteinase, it was of interest to see if the mutants were all defective in core protein precursor cleavage. Cleavage of the core protein precursors P4a, P4b, and P25K, products of



	Virus Alone-31C			Virus Alone-41C			Virus +pRb21-41C			Virus +pI7L-41C		
	Average	%	Fold Increase	Average	%	Fold Increase	Average	%	Fold Increase	Average	%	Fold Increase
Cts-16	2.2E+08	100%	418	5.2E+05	0.2%	1.0	5.3E+05	0.2%	1.0	2.8E+06	1.3%	5.3
Cts-34	8.3E+07	100%	137	6.1E+05	0.7%	1.0	5.9E+05	0.7%	1.0	1.7E+06	2.1%	2.9
Dts-4	2.2E+08	100%	108	2.0E+06	0.9%	1.0	1.7E+06	0.8%	0.8	4.2E+07	19.1%	20.7
Dts-8	1.8E+08	100%	134	1.3E+06	0.7%	1.0	1.1E+06	0.6%	0.8	1.4E+07	7.8%	10.5
Dts-35	3.6E+08	100%	195	1.9E+06	0.5%	1.0	2.3E+06	0.6%	1.2	3.7E+07	10.3%	20.1
Dts-93	9.5E+07	100%	74	1.3E+06	1.4%	1.0	1.0E+06	1.1%	0.8	1.1E+07	11.5%	8.5

Figure 2

Rescue of replication by plasmid born I7L. BSC_{40} cells were infected/transfected as indicated and incubated at the permissive (31C) or non-permissive (41C) temperature. At 24 hours after infection, the cells were harvested and the viral titer of the diluted cell lysate was determined. Fold increase was determined by dividing the titer by the titer of virus alone at 41C. % is the percentage of the viral titer at 31C. Bars = +/-1 standard error.

the A10L, A3L and L4R ORF's respectively, was initially assessed by western blot. BSC_{40} cells were infected at an MOI of 5, incubated at the appropriate temperature and harvested at 24 hpi. 100 μg/ml rifampicin (Boehringer-Manheim) and 8 mM hydroxyurea (applied one hour prior to infection) were used where needed. Cell pellets were resuspended in 50 μl of buffer and subjected to three freeze/thaw cycles. Aliquots of lysate were boiled with sample buffer and separated on 4–12% SDS PAGE gradient gels for P4a and P4b detection and 12% SDS PAGE gels for P25K detection. Membranes were incubated with a 1:1000 dilution of the appropriate polyclonal antibody, followed by a 1:2000 dilution of an anti-rabbit-HRP secondary antibody (Promega). Bands were visualized using the Opti-4CN detection system (BioRad). For all mutants, cleavage of P4a and P4b occurred at the permissive temperature but was absent or strongly reduced at the non-permissive temperature (Figure 3A). Cleavage of P25K at the AG*A site to produce 25 K did not occur at the non-permissive temperature, while a higher molecular weight band corresponding to the product created by cleavage at an AG*S site was present [17]. The banding patterns at the

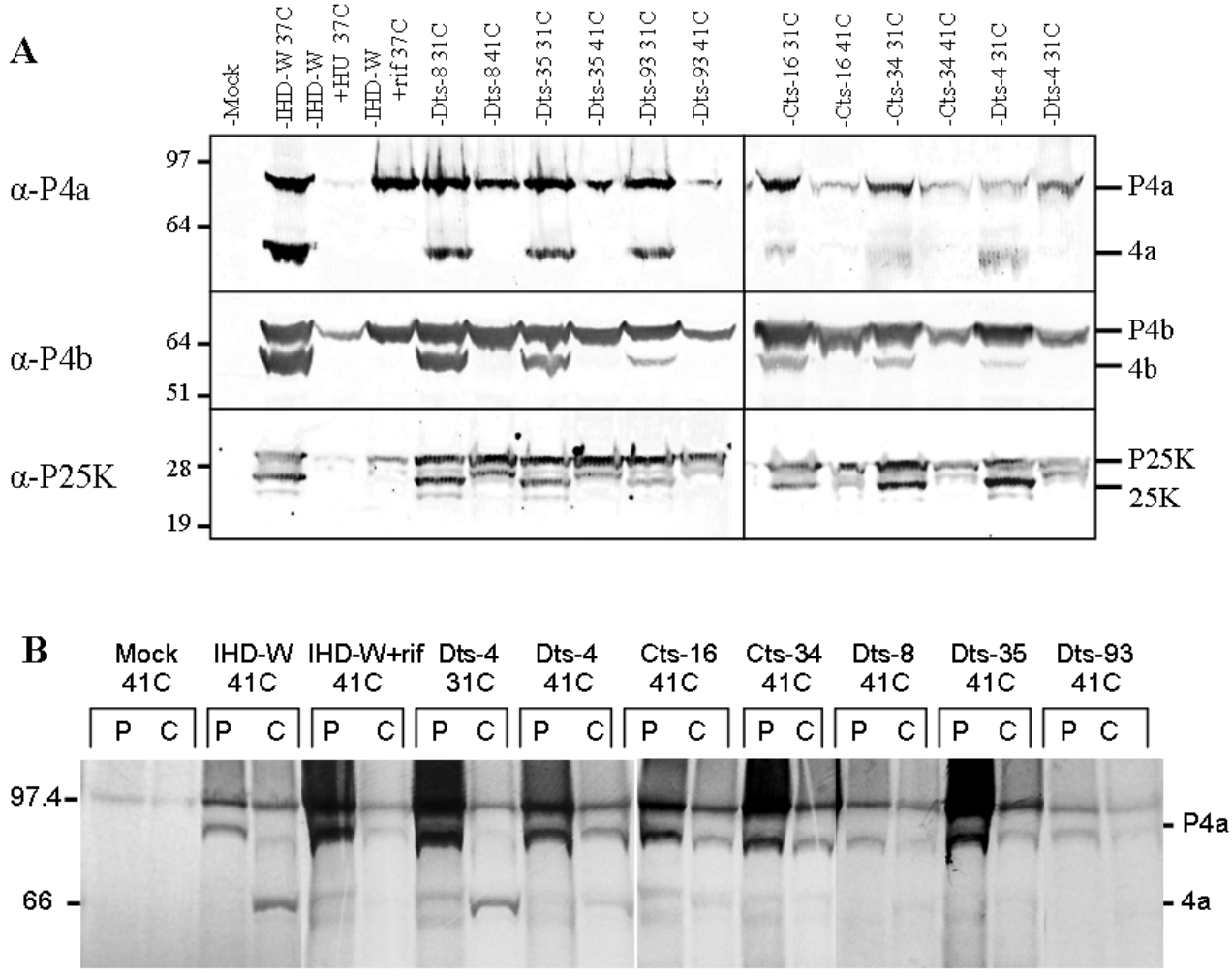


Figure 3

Analysis of core protein precursor processing at the permissive (31C) and non-permissive (41C) temperatures. (A) Infected BSC_{40} cells were incubated at the indicated temperature and harvested 24 hours after infection. Lysates were analyzed by Western blot using antisera against the indicated protein. (B) Infected BSC_{40} cells were labeled with [^{35}S]-methionine and [^{35}S]-cysteine for 45 minutes at 8 hours after infection. Cells were harvested after the pulse (P) and or after being chased (C) with unlabeled methionine and cysteine until 24 hours after infection. Immunoprecipitated samples were separated on a 4–12% SDS PAGE gradient gel. Rifampicin (rif) and hydroxyurea (HU) were used at final concentrations of 100 μg/ml and 8 mM respectively.

non-permissive temperature were similar to those seen in cells treated with rifampicin, a drug known to inhibit cleavage of core proteins [18]. Core protein precursor processing in both parental strains proceeded normally at 41C (data not shown).

The absence of cleavage at the non-permissive temperature was confirmed for P4a using pulse-chase immunoprecipitation. 100 mm plates of BSC_{40} cells were infected with virus at an MOI of 10 and incubated at 31 or 41C, as appropriate. At 8 hpi, the cells were labeled with 100 μCi of [^{35}S]-methionine and [^{35}S]-cysteine (EasyTag EXPRE^{35}S^{35}S; PerkinElmer) in methionine and cysteine free media. Rifampicin, where needed, was added at 100 μg/ml. After a 45 minute incubation, pulse wells were harvested, while chase wells were washed and treated with media containing a 100 fold excess of unlabeled methionine and cysteine and rifampicin if necessary. These were harvested at 24 hpi. Cell pellets were resuspended in 600 μl of RIPA buffer and subjected to three freeze/thaw cycles and sonication. Samples were centrifuged to remove debris and the lysate was incubated overnight with polyclonal antibodies against P4a followed by a second incubation after the addition of Protein A Sepharose beads (Amersham BioSciences). The washed beads were boiled in 20 μl of sample buffer and subjected to electrophoresis on a 4–12% SDS PAGE gel.

In all virus containing pulse samples, a strong band corresponding to P4a was clearly visible (Figure 3B). For IHD-W and Dts-4 at 31C, a representative example of the behavior of the ts viruses at the permissive temperature, the precursor containing band was strongly diminished after the chase period while a lower molecular weight band representing the cleaved product 4a appeared. At the non-permissive temperature, there was limited change in the intensity of the precursor and little or no cleavage product appeared. The pattern seen at the non-permissive temperature was similar to that of IHD-W infected cells treated with rifampicin.

The overall morphology of all six mutants was examined using electron microscopy. BSC_{40} cells were infected at an MOI of 10 and, after a one hour absorption period, incubated at 31 or 41C. Infected cells were collected at 24 hpi, fixed, embedded and stained. Dts-4 grown at 31C was examined as a representative of the ts mutants at the permissive temperature and was wild-type in its appearance. Both mature, brick-shaped particles with characteristic biconcave cores and spherical IV containing electron dense viroplasm were seen (Figure 4A–C). At the non-permissive temperature, all the ts mutants were similar in their microscopic appearance (Figure 4D–I). Normal crescent shaped membranes and IV were seen along with large numbers of defective IV. Many of the particles had asymmetrical condensation of the viroplasm, with the membrane sometimes collapsing on the empty side. Others formed dark, electron dense nucleoids. The appearance of these mutants is similar to what has previously been reported for Cts-16 [9,14], Dts-8 [13], and I7L conditional-lethals where I7L expression was inhibited by an operator/repressor system [6,8]. The appearance of Dts-35 differed from that reported by Dales [13], as particles with defective cores were not seen. However, Ansarah-Sobrinho and Moss also reported poorly formed cores in some of their I7L null mutants [6]. It seems then, that a deficiency in I7L can manifest itself in two different ways, with the virion morphology described here having been the most frequently observed.

Since the mutations in the I7L ts mutants fall into two distinct groups it is tempting to speculate that they might affect two different functions of I7L. Our results indicate that this is not the case, at least at the level of the virion formation, as all mutants were defective in the cleavage of core protein precursors and had their development arrested at a similar stage. Yet the possibility remains that the mutations affect two different elements required for proteinase function. The mutation in Cts-16 (and now Dts-8) at aa344 has been suspected, without proof, to inhibit protein cleavage by disrupting the arrangement of the catalytic triad due to its proximity to the cysteine residue at aa328. It is possible that the other mutants, with amino acid changes at the N-terminus of the protein between residues 29 and 37, may also sufficiently alter the structure or stability of the catalytic site to prevent proteolysis. However, because of their position this seems less likely. Instead, we suggest that the mutations occur within a region that constitutes a separate domain of unknown function that is necessary for I7L proteinase activity. Unfortunately the existing threading and homology model of I7L [15] does not include the 130 N-terminal most amino acids as this region does not fit any known structural domain.

Nevertheless, the properties of this region suggest several potential functions. One possibility is that the mutations disrupt the binding site of an unidentified co-factor(s) that I7L is believed to require as I7L produced in a cell-free translation system lacks cleavage activity [19]. The affected stretch of amino acids lies within a hydrophobic region [2], a common characteristic of sites of protein-protein interaction. The mutations also lie within a region that shows weak homology to the type II DNA topoisomerase of *Saccharomyces cerevisiae* [9], raising the possibility of a nucleic acid binding site. Adenovirus proteinase, an I7L homolog, requires both a peptide and a DNA cofactor for full activity [20]. Alternatively, the mutations may interfere with a potential regulatory cleavage as I7L contains two AG*X motifs at its N-terminal end

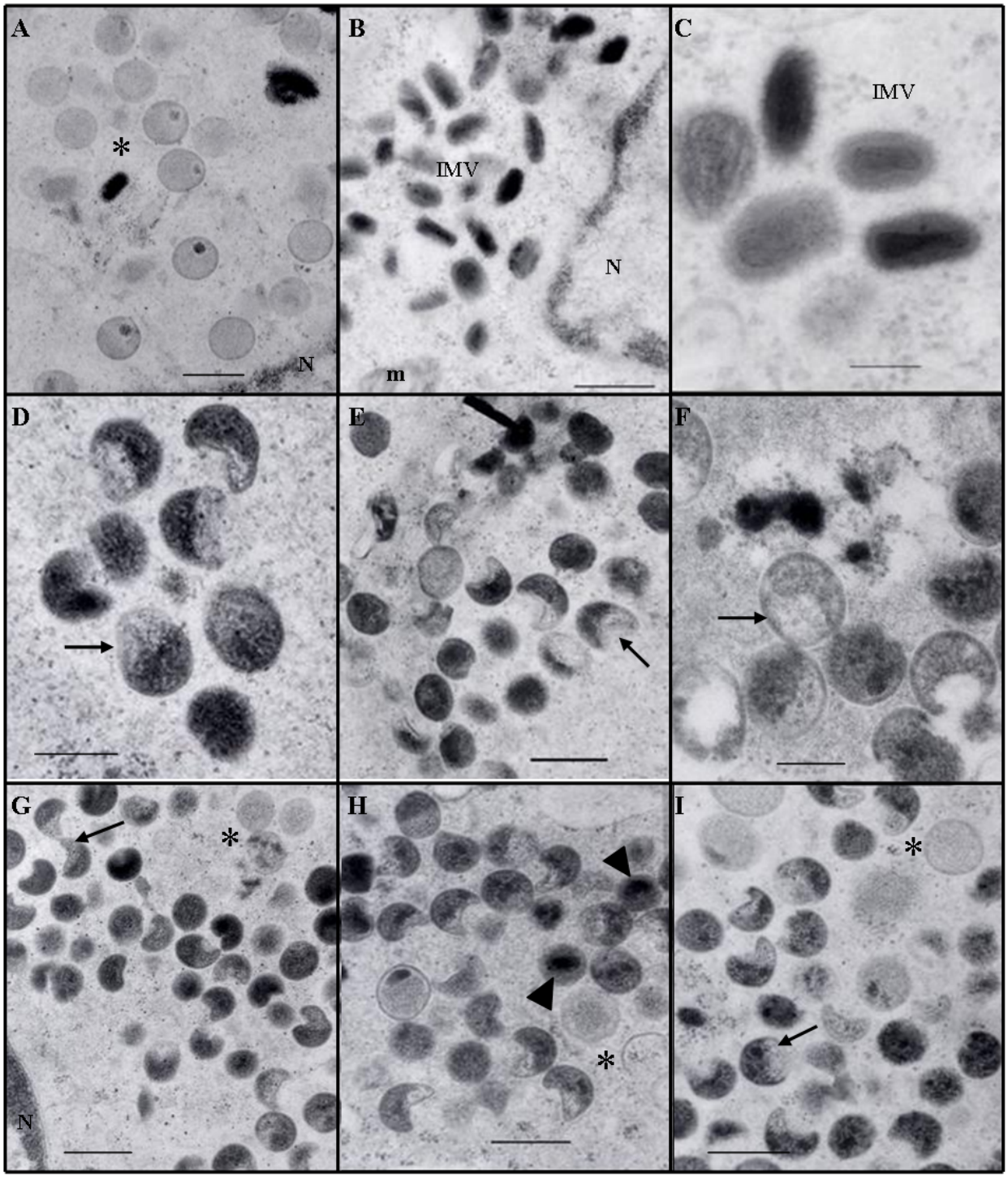


Figure 4

Electron micrographs of virus infected BSC_{40} cells. MOI = 10 and cells were harvested and fixed 24 hours after infection. Dts-4 at the permissive temperature of 31C (A-C). Dts-4 (D), Cts-16 (E), Cts-34 (F), Dts-8 (G), Dts-35 (H) and Dts-93 (I) at the non-permissive temperature of 41C. Bars represent 400 nm except in C, D and F (bar = 200 nm). N, nucleus; m, mitochondria; IMV, intracellular mature virion; asterisk, immature viral particle; arrow, representative particles with asymmetrical viroplasm condensation; arrow head, nucleoids.

and third at its C-terminal end. One of these AG*X sites is directly disrupted by the mutation in Cts-34 (AGL to AEL)

It is important to note that until more detailed structural and biochemical information about I7L is available, any conclusions about the processes disrupted by the mutations within these ts mutants are tentative. However, their location provides a starting point in the search for regions of I7L important to its activity.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MJM conducted the experiments and wrote the manuscript. CMB assisted with the sequencing and edited the manuscript. DEH conceived the study, coordinated the research efforts and edited the manuscript. All authors read and approved the final manuscript.

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